TECHNICAL UNIVERSITY OF LIBEREC
Faculty of Mechatronics, Informatics
and Interdisciplinary Studies

# COMPARISON OF DIFFERENT ANALYTIC METHODS FOR STUDYING OF TOXICITY OF NANOMATERIALS

**Bachelor thesis**

*Study programme:* B3942 – Nanotechnology
*Study branch:* 3942R002 – Nanomaterials

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Liberec 2015

TECHNICKÁ UNIVERZITA V LIBERCI
Fakulta mechatroniky, informatiky
a mezioborových studií

# POROVNÁNÍ ANALYTICKÝCH METOD PRO STUDIUM TOXICITY NANOMATERIÁLŮ

**Bakalářská práce**

*Studijní program:* B3942 – Nanotechnologie
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# ZADÁNÍ BAKALÁŘSKÉ PRÁCE

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1. Vypracovat literární rešerši na téma: možnosti detekce toxicity nanomateriálů na nanočásticích s použitím mikroskopických řas (Zdrojem informací budou především vědecké články dostupné na běžných vyhledávačích: Web of Science, Scopus, Google Scholar).
2. Vybrat nejméně tři typy nanočástic, jejichž potenciální toxicita bude testována pomocí nejméně třech analytických metod, jako je např. stanovení změny koncentrace chlorofylu a detekce efektivity fotosystému II (Stanovení quantum yield).
3. Vybrat vhodný modelový mikroorganizmus - mikroskopickou řasu na základě literární rešerše.

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**[3] I. Linhart: Toxikologie: Interakce škodlivých látek s živými organismy, jejich mechanismy, projevy a důsledky. VŠCHT Praha (1. vydání, 2012)**

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# Abstract

The bachelor thesis deals with properties and analytic method for studying nanotoxicity of different nanoparticles on model organisms. Main aim of experimental part was comparison of three different analytic methods: determination of efficiency of photosystem II, determination of concentration of chlorophyll *a* and detection of damage of cell membranes of green alga *Chlamydomonas reinhardtii*; for studying nanotoxicity of three nanoparticles: nanodiamond, magnetite and nZVI. Nanoparticles were characterized using the DLS and DCS methods. Toxicity experiments were statistically evaluated using two-way ANOVA. Each of the analytical method was compared with an independent article and has its advantages and disadvantages that are discussed in the thesis. The best method was found to be the determination of concentration of chlorophyll *a* due to its precise, fast and solid stable results.

## Key words:

Nanotoxicity, nanodiamond, nZVI, magnetite, Quantum yield of photosystem II, Sytox Green, chlorophyll *a*, Chlamydomonas *reinhardtii*, DLS, DCS

# Abstrakt

Bakalářská práce pojednává o vlastnostech a analytických metodách pro studium nanotoxicity různých nanočástic na modelových organismech. Experimentální náplní práce bylo srovnání tří analytických metod: stanovení účinnosti fotosystému II, určení koncentrace chlorofylu *a* a detekce poškozených buněčných membrán u zelené řasy *Chlamydomonas reinhardtii*; pro studium nanotoxicity tří nanočástic: nanodiamant, magnetit a nZVI. Nanočástice byly charakterizovány pomocí metod DLS a DCS. Výsledky experimentů byly statisticky zhodnoceny použitím two-way ANOVA. Každá z analytických metod byla srovnána s nezávislým článkem a má své výhody a nevýhody, které jsou diskutovány v této práci. Nejlepší metodou bylo shledáno určení koncentrace chlorofylu *a* kvůli svým přesným, rychlým a stabilním výsledkům.

## Klíčová slova:

Nanotoxicita, nanodiamant, nZVI, magnetit, Kvantový výtěžek fotosystému II, SYTOX Green, chlorofyl *a*, *Chlamydomonas reinhardtii*, DLS, DCS

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# List of Abbreviation

DCS – dynamic light scattering

DLS – differential centrifugal sedimentation

DMSO – dimethyl sulfoxide

dsRNA – double-stranded RNA

FITC – fluorescent isothiocyanate

MTT – 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide

NADH, $NAD^+$ – oxidized and reduced form of nicotinamide adenine dinucleotide

NP, NPs – nanoparticle, nanoparticles

nZVI – nanoscale zero-valent iron

OD – optical density

QY, $\Phi_{II}$ – quantum yield of photosystem II.

RI – refractive index

XTT – 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide

# 1 Introduction

Nanotoxicology is one of the most important parts of nanotechnology. It is a sub-discipline of nanotechnology, which studies the physical and chemical interactions between nanoparticles (NPs) and biological systems. Even the smallest differences in composition, size, shape or surface chemistry could be responsible for different toxic effect on biological system (Albanese, Tang, Chan 2012). The particles are so small that they could be equal to the size of natural proteins, and because of that, they could reach places where any larger particles could not get. For example throw the cell wall to the nucleus. Moreover the high surface/volume ratio is an important parameter. Because of high specific surface area of NPs, there is a high probability that other biological components can interact with them (Fischer, Chan 2007).

Furthermore, there are problems in using toxicological tests in nanotoxicity research. Toxicological test could be used, but we have to be aware of different behavior, chemical and physical interactions of NPs. Therefore, it is necessary to study not only the effect of nanoparticles on organisms, but also behavior of NPs during testing.

A goal of this thesis is a comparison of three different analytical methods - Determination of efficiency of photosystem II, Detection of damage of cell membranes and Determination of the concentrations of chlorophyll *a*, for detection of toxicity of nZVI, nanodiamond and magnetite nanoparticles using model organism – alga *Chlamydomonas reinhardtii*.

# 2 Literature Overview

## 2.1 Definition of Nanoparticles

Nanoparticles are particles of any shape or chemical composition that have at least one dimension within the range of 1 – 100 nm (Environment DG 2015). This dimension is approximately 1000 times thinner than human hair. We can see comparison of different particles, molecules and other microscopic objects in Figure 2.1.

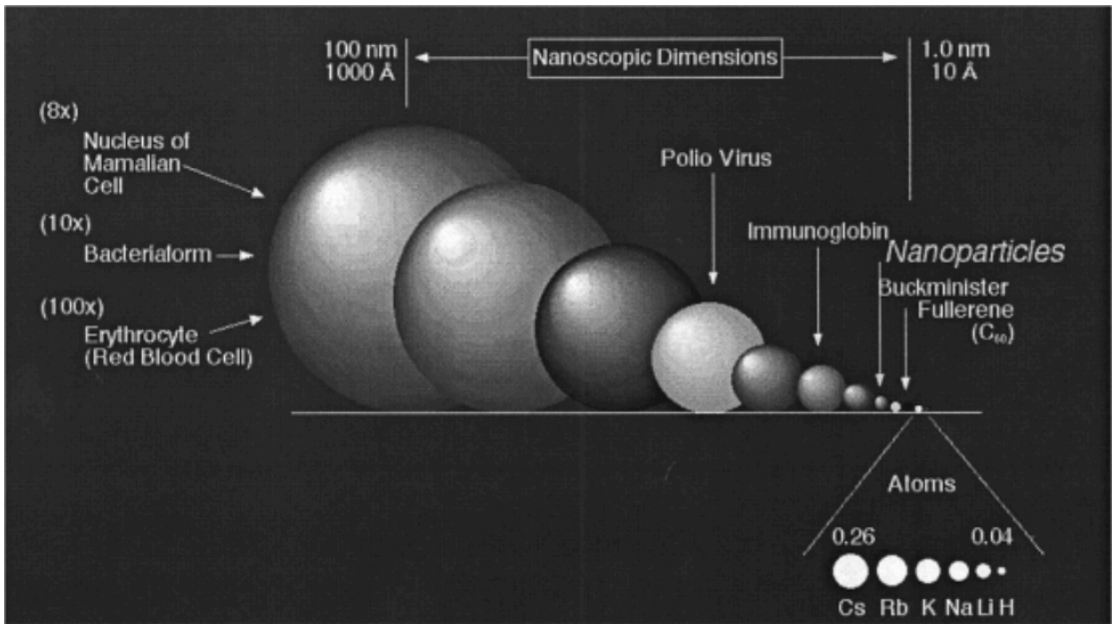


*Figure 2.1: Graphical representation of comparison of nanoparticle size.* (Klabunde 2001)

Historically, nanoworld was recognized by Richard Feynman who had a lecture - *There's Plenty of Room at the Bottom*, in December 1959. In this lecture, Feynman spoke about the problem of manipulating and controlling things on a very small scale. Even though this lecture went unnoticed by wide public, it was rediscovered in 1990s and was considered as one of the first hint of nanotechnology.

The name *nano* (from Greek nânos – dwarf) had become fashionable in 90s of the last century (Couvreur et al. 1990; Tröster, Müller, Kreuter 1990; Seiji et al. 1990), but the research about these small particles started much earlier. We can find studies from 70s – 80s which were focused on *nanoparticles*, but the authors used different name - *ultrafine particles* (Granqvist and Buhrman 1976).

## 2.2 Properties of nanoparticles

Nanoparticles (NPs) and nanostructures are so small that quantum effects, properties of chemical bonds and atomic powers, strongly influence their behavior (Nel 2006). The change of chemical composition, size, shape or surface composition could change physical, chemical, mechanical and other properties of the nanoparticle (Albanese, Tang, Chan 2012).

NPs have enormous specific surface area – nanoparticle with shape of ball and radius of 2.5 nm and density of 5 $g/cm^3$ has surface equal to 240 $m^2/g$ (Borm et al. 2006). Therefore, NPs have a high active surface, however, the surface of NPs is not simply "naked". There is an adhesive force, which cause agglomeration between these NPs. With increasing size of agglomerates, the surface is decreasing and NPs are losing their "special" ability. One of the methods to avoid undesirable agglomeration is the surface modification of NPs by suitable chemical substances (Kango et al. 2013; Bagwe, Hilliard, Tan 2006).

Chemically identical particles can change colour of suspension due to their different size and shape. This happens because the area of photons absorption is changing with the size of particles (Jain et al. 2006).

Furthermore, structural composition strongly influences properties of NPs. Structures smaller than 100 nm exhibit different mechanical properties than larger ones. Differences could be in strength, for example carbon nanotubes are 100-times stronger than steel and 6-times lighter, hardness - some NPs are responsible for improving hardness and durability of metals, ductility and superplasticity (Hájková 2011).

The differences are not only in mechanical properties but also in magnetic, electric and optic fields. Magnetic NPs exhibit dramatic changes in some of their magnetic properties. Because of the large surface and quantum size effects NPs exhibit superparamagnetic phenomena and quantum tunnelling of magnetization (Mathew, Juang 2007).

Also the changes in melting temperature of metals are a function of the size of particles. For example the difference of melting point between gold in solid state and in a form of powder material with a size of particles around 2 nm is around 1000 °C (Buffat, Borel 1976).

## 2.3 Areas of applications of NPs

There are lots of applications for nanoparticles in various sectors like medicine, manufacturing and materials, energy, electronics, environment and many others. I will briefly summarize some of the most interesting and important applications of NPs in the field of environment (nZVI) and biomarkers (nanodiamond), because this field is closest to my thesis.

One of the most important applications of NPs and nanomaterials is in decontamination water and soil systems via remediation. From the full range of NPs, nanoscale zero-valent iron (nZVI) is the most suitable and the most frequently used one (Wang, Zhang 1997). It is environmentally safe, because iron is natural part of the environment. nZVI functionality is based on the fact, that it is strong reducing agent. Zero-valent iron is a donor of electrons and the reduced compounds are the acceptors of electrons. This implies that a lot of different environmental contaminants could be reduced by oxidation of zero-valent iron (mechanism in Figure 2.2).

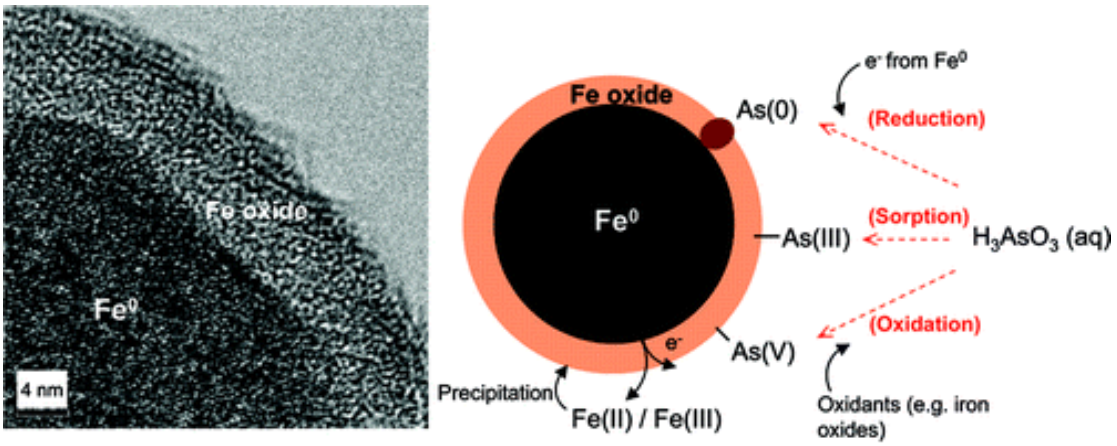


*Figure 2.2: Left: core-shell structure of nZVI, Right: scheme of nZVI and with it associated reactions with contaminants* (ACS 2009).

Studies have shown, that nZVI is very effective in removing *in situ* contaminants such as PCE – perchlorethane, TCE - trichlorethane, tetrachloromethane, DDT

(Wang, Zhang 1997; Song, Carraway 2005), TNT – trinitrotoluene (Zhang, Lin, Chen 2009; Zhang et al. 2010) and heavy metals like chrome and lead (Klimkova et al. 2011) from underground water and soil aquifers. Even better results can be achieved using palladized nanoscale iron particles (Lien, Zhang 1999).

However, the efficiency depends not only on the reactivity of nZVI with the contaminants, but also on the ability to deliver particles to correct spot and ensure contact with contaminants. nZVI could be applied for example at the locality where are rocky or clay layers by an injection of aqueous suspensions with concentrations in order of units of grams per litre (Černík et al. 2010). The important parameter, which influences migration capability of the NPs, is their size distribution over time. nZVI is very reactive and has high tendency to aggregate and create bigger particles (Černík et al. 2010). These aggregates could reach dimension of several micrometres or even more. With increasing size of particles is their surface decreasing and, therefore, is decreasing their reactivity and mobility in specific environment. Aggregates of particles are created mainly by Van der Waals powers and by magnetic interactions. Numerous studies have been dealing with this problem and trying to find solution. One of the solutions is the modification of nanoparticle surface. This could be done for example by electrostatic stabilization, steric stabilization, electrosteric stabilization or we can use stabilizing agents such as anionic surfactants, polyacrylic acid (PAA), triblock copolymers (PMAA-PMMA-PSS) and so on (Černík et al. 2010). For nZVI is a great stabilizer starch and CMC (sodium carboxymethylcellulose). CMC is commonly used for NPs of Ag and iron oxides. Both stabilizers are cheap and environmentally friendly. Unstabilized NPs are creating agglomerates in several minutes, but NPs stabilized by CMC or by starch are in water dispersed for several days. Tests showed, that these stabilizers also increase speed and mount of eliminated contaminants (for example TCE – trichloroethylene). Other good stabilizer for NPs of iron is biodegradable polyacrylic acid (PAA)(Kanel et al. 2008).

Fast and accurate detection of microorganism is important in many ways (purity of drinking water and food, clinical diagnoses and therapies and also because of bioterrorism) (Zhao et al. 2004).

Another application of NPs is as cellular biomarkers. For this we can use nanodiamond, which bright fluorescence at 600 nm to 800 nm wavelength via nitrogen-vacancy point defects after irradiation by high-energy ion beam and thermal annealing (Yu et al. 2005). We can obtain high concentrations of nitrogen-vacancy centres and see individual (~35 and ~100 nm) diamond crystallites because of irradiation and thermal annealing process using fluorescence microscope (Fu et al. 2007). The advantages of this nanomaterial are long-term photostability, no fluorescence blinking, minimal interference with cell autofluorescence, facile surface modification and relative size-independent properties (up to 100 nm) (Fu et al. 2007). Combination of all these unique properties give us a perfect tool for single-particle tracking in heterogeneous environment, even in human cells (Liu et al. 2007).

## 2.4 Toxic effects of nanoparticles

Numerous studies report about toxicity of nanomaterials on different organism from microorganism such as bacteria or algae, to mammals (Li, Zhang, Yan 2014). This branch is very wide and contains huge amounts of information. If we want to study specific toxic effects of NPs, we need to have precise information about their properties and also information about the organism, which we want to test, because the toxic effect can vary depending on these factors.

The most studied nanomaterial regarding toxicity has been silver. Even old Romans knew about antibacterial effects of silver. Metallic silver is inert and passes through human body. Because of that it is better to use silver oxide $Ag_2O$. Positively charged ions $Ag^+$ are highly toxic and fatal for microorganism such as bacteria (Choi, Hu 2008), algae (Dash et al. 2012), fungi and yeast (Kvitek et al. 2011). These ions act in several different ways. Silver can attack places inside cells and deactivates important physiological functions such as cell wall synthesis, transport through membrane, synthesis and translation nucleic acid. All this leads to cell death. Advantage in using NPs of silver (in dimension 5 – 15 nm) is that they have high specific surface area. Silver can be formed in humid conditions, for example in body fluids, due to oxidation process on the surface of particle. This reaction runs very slowly, which causes long duration of his effect (Prnka, Šperlink 2006).

Silver NPs are not the only one, which are toxic. It was confirmed in several studies that one of the most toxic particles in general are copper oxide (CuO) and zinc oxide (ZnO). The toxicity of these NPs and also bulk materials were tested on several organism such as microalga *Pseudokirchneriella subcapitata* (Aruoja et al. 2009), protozoa *Tetrahymena thermophila* (Mortimer, Kasemets, Kahru 2010), yeast *Saccharomyces cerevisiae* (Kasemets et al. 2009) and bacteria *Escherichia coli*, *Bacillus subtilis* and *Streptococcus aureus* (Baek and An 2011). Toxic effects were proved in different scale for all of these organisms, whereas each of the tested organisms showed different response.

The toxicity of nanomaterials is not always undesired and we can use it for improving lots of technologies such as cleaning water systems (dams, water purifiers, filters etc.) and bactericidal materials (Sondi, Salopek-Sondi 2004).

### 2.4.1 Organisms used for nanotoxicity assessment

*Why we are using model organisms?* The primary reason is that all organisms are in some way similar (similarity of genetic code) and share a bit of relatedness. Due to this we are able to predict toxic effect on human and others organism by studying the process in model organisms. For example chimpanzee is our closest living evolutionary relative (Consortium 2005), so studying them have a great potential in understanding mechanism of toxic effect. However, chimpanzees are rarely used in research and they are protected from highly invasive procedures. Next very relative organism to humans are rodents, which have still more similarities than differences (Chinwalla et al. 2002). Because of that rodents are commonly used in biological research (Wasserman et al. 2000).

Therefore, it is important to choose appropriate organism for research, which we want to conduct. We also should take into account that each nanoparticle have heterogeneous composition and different properties, thus the study of effects on living organisms has to be equivalent to the type of NPs and their applications (Griffitt et al. 2008).

Generally, we can rank model organisms for toxicology into several groups, such as microorganisms, small crustaceans, worms, fish, mammals and plants, depending on their size and type of organism for toxicity assessment.

Bacteria and algae are usually used as model microorganisms. The prokaryote model systems often represent *Escherichia coli* (Baek and An 2011), which is a common constituent of human digestive system, *Bacillus subtilis* (Guérout-Fleury et al. 1995), which is widely used in biotechnology, *Staphylococcus aureus*, which is used in a antibacterial assessment (Shahverdi et al. 2007) and many other.
Representatives of microalgae model are *Pseudokirchneriella subcapitata*, which is often used in nanotoxicity assessment (Muyssen, Janssen 2001; Youn et al. 2012) and as well *Chlamydomonas reinhardtii* (Navarro et al. 2008) and *Chlorella* (Ji, Long, Lin 2011). Algal model organisms are usually single cell species living in freshwaters.

*Cladocera* are small crustaceans, which are commonly known as water fleas. We can mention *Ceriodaphnia dubia* as frequently used model organism, which is useful for standard toxicological studies because it represents a simple model for food transfer (Bouldin et al. 2008). Other well-known crustaceans are *Daphnia pulex* and *Daphnia magna*, which are widely used to detect contaminants in water (Lovern, Klaper 2006). Daphnids are filter-feeders and because of that they could be more prone to NPs exposure than fish (Griffitt et al. 2008).

One of the most common organisms, which live in soil on all continents except Antarctica, is an earthworm *Eisenia fetida*. *E. fetida* is important because of decomposition of organic waste in soils, and therefore it is used as model organism, for example, to find toxicity of nanomaterials in application in common soil systems (Shoults-Wilson et al. 2011). Likewise earthworms, nematodes are important in the soil food web. One of them is *Caenorhabditis elegans*, a simple multicellular eukaryote, which is used as a superior model in genetics, neurobiology, developmental biology and nanoecotoxicology assessment (Wang, Wick, Xing 2009; Zhang et al. 2012).

All these model organisms are rather simple, which is advantageous for their handling and maintaining in culture. However, we can find suitable organisms for toxicological research even within vertebrates. Some of these are *Danio rerio* (well known as Zebrafish) (Griffitt et al. 2007) and *Pimephales promelas* (also known as Fathead minnow) (Zhu, Oberdörster, Haasch 2006). *D. rerio* is a fish with length around 2.5-3.8 cm and well known behaviour, because of increasing number of research studies (Hill et al. 2005). In the case of *D. rerio* adults specimens are usually used, but in the case of *P. promelas* embryos are preferred (Laban et al. 2010). Both fish are well documented and used in more sectors such as toxicology research (King-Heiden et al. 2009), biomedicine and molecular genetic.

Principally, the development and application of NPs and nanomaterials is growing in agriculture. Therefore it is important to study toxic effects on plants that are normally used as food and with which we can get in touch (Monica, Cremonini 2009). Higher plants could be used for nanotoxicity assessment common examples are *Raphanus sativus* (radish), *Brassica napus* (rape), *Lolium multiflorum* (ryegrass), *Lactula sativa* (lettuce), *Zea mays* (corn), *Cucumis sativus* (cucumber) and many more. Seed germination and root growth are usually examined (Lin, Xing 2007; Larue et al. 2014; Wang et al. 2011).

### 2.4.2 Analytical methods of nanotoxicity assessment

*In vitro* tests are very popular in the nanotoxicity assessment. *In vitro* (from lat. "in glass") means that we are working in artificial conditions in the laboratory. These tests have a lot of advantages such as: small amount of testing materials is required, amount of produce of toxic waste is limited, methods are usually fast, cheap and easily reproductive, and mainly the environment and testing conditions are under control (Takhar, Mahant 2011).

It is not the objective of this diploma work to write an exhaustive review of methods and techniques of analytical nanotoxicity assessment. Rather than that I will briefly describe some of techniques, which are available and currently applied in the field of nanotoxicology for *in vitro* experiments.

Generally, assessment techniques of *in vitro* experiments are divided into two areas: cell viability such as ratio of live/dead cells, growth rate, proliferation, apoptosis and necrosis; and toxicity mechanism such as oxidative stress and DNA damage detection techniques (Marquis et al. 2009).

One of the most widespread proliferation assays used is cellular reduction of tetrazolium salts to producing formazan dyes (tests such as MTT, XTT), that are detectable by optical absorbance and used as indicator of cell metabolism (Marquis et al. 2009; Wang, Yu, Wickliffe 2011). Notable advantage in using this method is that it requires a minimal physical manipulation of the model cells and gives quick and reproducible results. On the other side, the understanding of assays results could be misleading, for example because of unclear mechanism of cellular reduction, which is located outside of the mitochondria (Marquis et al. 2009), or because of the reaction of tetrazolium salts with NPs (for example superoxide $TiO_2$) (Wang, Yu, Wickliffe 2011).
Another assays that are based on metabolism of model cell, are [$^3$H]thymidine incorporation and Alamar Blue, known as *resazurin* test. [$^3$H]thymidine is a sensitive method for detection of cell proliferation, because radioactive-labelled thymidine is incorporated in freshly synthetized DNA (Takhar, Mahant 2011). The disadvantage is in high price and possible *in vitro* toxicity, which cause frequent avoidance of this method. Alamar blue reduction test is measure of cellular reduction potential and has been used for more than 50 years to detect bacteria, yeast contamination of milk and so on (O'Brien et al. 2000). Alamar blue, which is blue and non-fluorescent, is reduced in viable cells to soluble resorufin, which is pink and highly fluorescent. However, the biochemical mechanism of this reaction is still not known (O'Brien et al. 2000). Problem with Alamar blue is that it could react with NPs and by this way the result can affected (Zhou et al. 2010).

Other assays use membrane integrity as a tool to determine cellular viability. Within this group belong methods either using the uptake of supravital dye (Trypan Blue, Neutral Red, propidium iodide assays) or the entering of active enzyme into cell (lactate dehydrogenase assay, LDH). Advantage is that all these techniques are highly reproducible (Marquis et al. 2009).

Trypan Blue and propidium iodide are charged molecules and do not enter freely inside the unaffected cell. However, both dyes can get inside if the cell has damaged membrane (Hillegass et al. 2010). Trypan Blue stains cell with a absorption around 605 nm (Hauck, Ghazani, Chan 2008) and propidium iodide stains DNA and double-stranded RNA, where it fluorescents around 617 nm (Jan et al. 2008; Marquis et al. 2009). Neutral Red is a little bit different. It is uncharged and it could enter either alive or death cells, but if it gets inside the living cells, it absorbs at 540 nm due to protonation by acidic lysosomes because of pH change (Shukla et al. 2011).

LDH (lactate dehydrogenase) is an oxidoreductase, which is present in a wide variety of organisms. It catalyses the interconversion of pyruvate and lactate with concomitant interconversion of NADH and $NAD^+$. If toxic material damages tissue or cell, cells release LDH into bloodstream. Since the fact that LDH is a stable enzyme, it could be identified in higher levels. The colorimetric LDH assays are based on the reduction of MTT in a NADH-coupled enzymatic reaction to a reduce form of MTT, which exhibits an absorption around 565 nm (Akhtar et al. 2010).

Furthermore, it is very important to find out any possible interference between NPs and assay components, because if any exists, it could degrade the accuracy of assay. Unfortunately, this interference could not be predicted by interactions between NPs and assay components (Ong et al. 2014).

We can combine the necrosis assays with apoptosis detection assays to get a better picture of a cell death due to action of NPs. These assays include inspection of morphological changes, the TUNEL assay (Sharma et al. 2012), the COMET assay (Barnes et al. 2008), the annexin-V assay (Foldbjerg et al. 2009) and DNA laddering (Ye et al. 2010, p. 2).

The TUNEL assay is using double-strand breakage and DNA fragmentation during apoptosis. Usually, DNA polymerase is used along with 5-bromo-2deoxy-uridine (BrdU), which are incorporated into repaired double-strand breaks in cells. It is then possible to detect double-strand breaks in nuclear DNA in a microscope anti-BrdU antibody that are used for labelling DNA containing BrdU.

The most frequently used DNA damage assay is COMET assay. Cells embedded in agarose gel are lysed with detergent and high salt to formed nucleoid (structure in

which DNA in concentrated). Thereafter the nucleoids are labelled by ethidium bromide and separated using the electrophoresis. The DNA damage is indicated by the amount of DNA fragments using fluorescence microscope.

The annexin-V is a phosphatidylserine-specific binding substrate. It is using exposed phosphatidylserine, which is located on the exterior of the cell during apoptotic restructuring of membrane. The annexin-V could be labelled by a fluorescent dye, such as FITC, to highlight the membranes in early and late state of apoptosis. Due to that this technique is usually taken as an apoptosis assessment.

The oldest DNA damage assay technique is DNA laddering, which is using gel electrophoresis to detect DNA damage. DNA fragmentation is isolated and labelled by fluorescent dye from cells, which were exposed to a potential toxicant (Marquis et al. 2009).

Inspection of morphological changes is one of the cheapest and the least complicated method, which requires only a light microscope and visual inspection. This method, despite all of these advantages, is used less than others, because of time demands and possible mistake made by human-factor (Marquis et al. 2009).

To conclude, there are many methods for nanotoxicity assessment, nevertheless, we must be always aware about the potential limits of their application.

# 3 Material and Methods

## 3.1 Nanoparticles and their characterization

I used three types of NPs: nanoscale zero-valent iron (nZVI, batch 197 obtained from Nanoiron Ltd. Company, Czech Republic), magnetite nanoparticles ($Fe_3O_4$, batch ZH09A obtained from Palacky University Olomouc, Czech Republic) and nanodiamond (cubic diamond, obtained from International Technology Center, USA).

I prepared stock of NPs suspensions always fresh before further tests. The concentration of stock suspension was 10 g/L, therefore, I weighted 0.05 g of each NPs and then put it into flask with 10 mL of deionized water (DI). NPs in DI water were then dispersed using a mixer (Miccra D-9 Homogenizer, Germany) for 10 minutes to avoid problems with pipetting incorrect concentration of NPs.
I characterized NPs in DI water and WC medium, which I used for algal cultivation. DI water was used for comparison with WC medium. I prepared three concentrations of NPs from suspension stock – 0.01 g/L; 0.05 g/L; 0.5 g/L.
I mixed each sample for 15 seconds with minishaker (lab dancer, IKA) before every measurement.

NPs were characterized using disc centrifuge (model DC24000 UHR, CPS Instruments, UK) (Figure 3.3) at first. This method uses differential centrifugal sedimentation (DCS). The principle of separation of particles by size is based on centrifuge sedimentation in a liquid medium (Figure 3.1). The sedimentation is than stabilized by density gradient, which is made by liquid medium, usually sucrose.

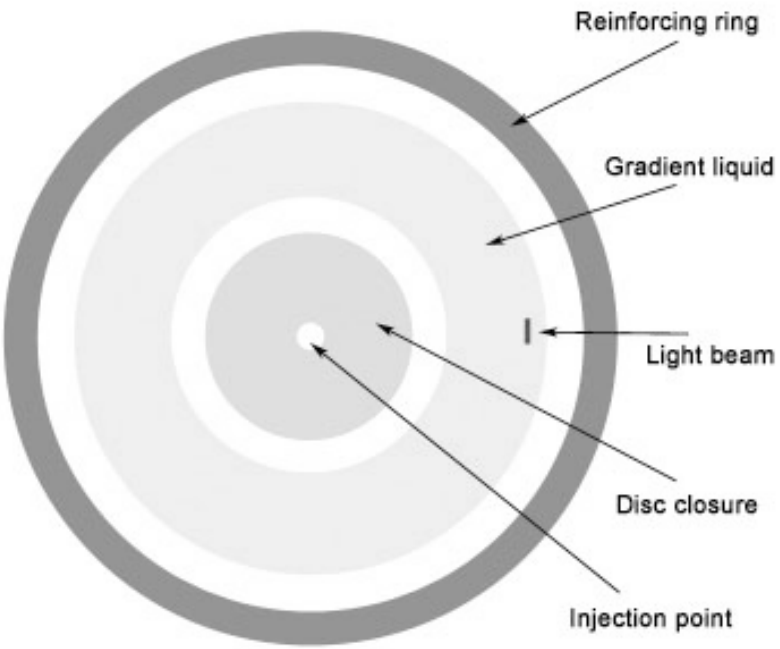


*Figure 3.1: Front view of the disc with the sample injection point in the middle of the instrument* (CPS 2013)

After an injection of particles in the middle of rotating disc they gradually sediment. On the edge of rotating disc is a light beam, which passes through the disc (Figure 3.2).

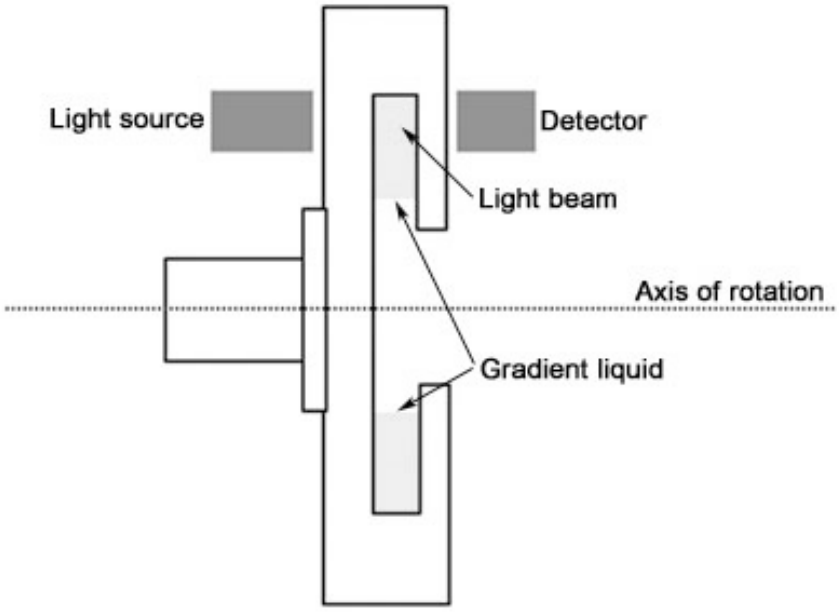


*Figure 3.2: A side view of the disc* (CPS 2013)

When the particles reach the outside edge of rotating disc, they scatter or block the light beam. The intensity of light, which passes through the disc is continuously measured and thereafter converted to the particle size distribution using operating software.

The size range depends on the density of measured particles. With high density (6 times the density of water) the maximum and minimum size are smaller (from 10 μm to under 0.005 μm) otherwise with low density (approximately 0.85–1.10 g/cm$^3$) is the maximum and minimum higher (from 75 μm to 0.02 μm).

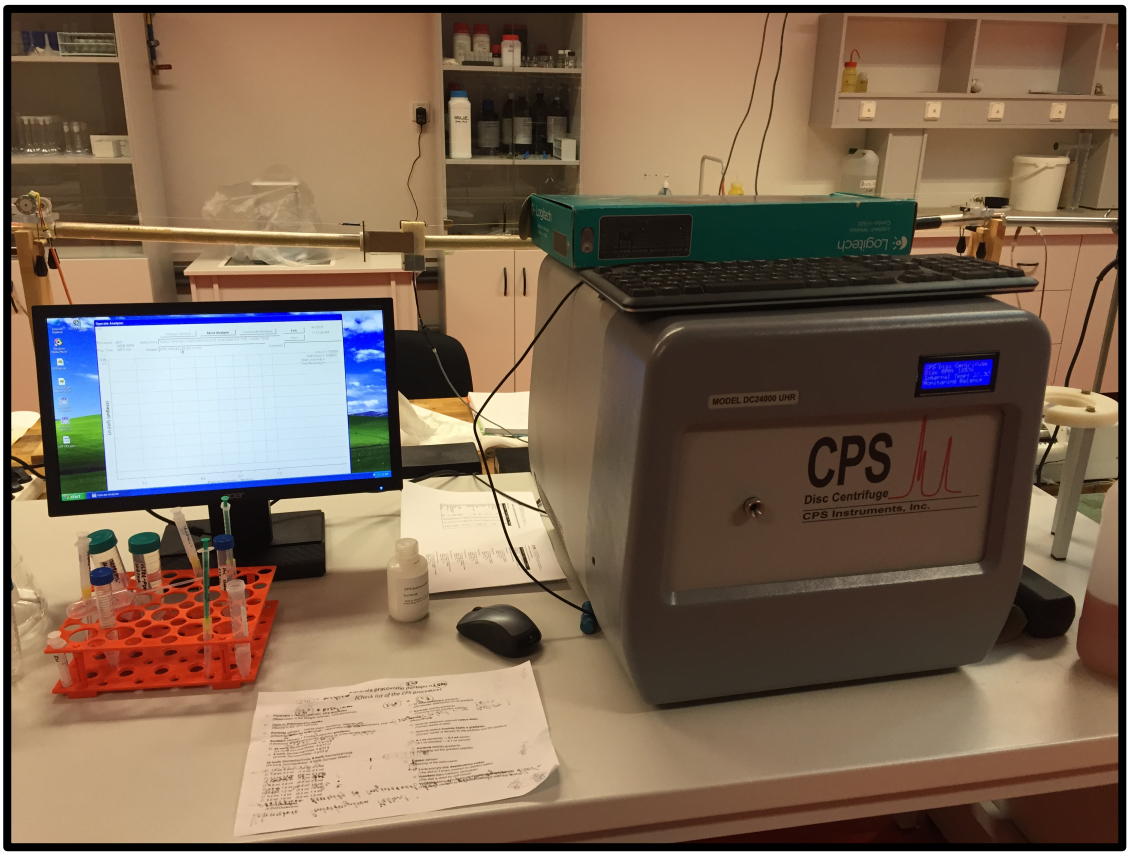


*Figure 3.3: Disc centrifuge (model DC24000 UHR, CPS Instruments)*

The total volume of sample (for example concentration 0.01 g/L) needed for three-day experiment was 2 mL; due to the fact that disc centrifuge requires only 100 μL of sample per one test. Afterwards I adjusted settings of disc centrifuge. Rotational speed was set at 8000 RPM. I used 24% and 8% sucrose for setting up the density gradient and 0.5 mL of dodecane to slow down evaporation. The chosen procedure was based on the type of NPs – differences were in settings of density, absorption and refractive index (RI)**:** nZVI – density was equal to 5.24 g/mL, absorption to 0.1

and RI to 1.7; nanodiamond – density equal to 3.515 g/mL, absorption to 1 and RI to 2.42; magnetite NPs – density was set to 5.24 g/mL, absorption to 0.5 and RI to 1.7. The disk centrifuge was calibrated with a poly(vinyl chloride) latex before each test with a mean weight particle diameter of 476 nm. I recorded raw data such as mean weight of NPs diameter and the polydispersity index of NPs using CPS software. Mean weight particle size is based on the fact, that measured particles are spherical shape and we know the particle density. The diameter of the sphere has than same weight as the measured particle.

The second method is based on dynamic light scattering (DLS) for particle size characterization and was measured by Zetasizer (Nano Series, Malvern Instruments) (Figure 3.4).


*Figure 3.4: Zetasizer (Nano series, Malvern Instruments)*

The principle of this method is based on DLS. Sample is exposed to laser beam and the light from laser is then scattered on suspension particles. The scattered light is sensed using the detector, which is in specific angle to the scattered light. In this

case is best choice 173° because of several reason. For example the laser beam passes a smaller sample volume, so it is possible to measure higher concentration. Moreover, there is no multiple scattering of the light. Another reason is that you avoid the dust detection, because dust is composed from large particles and the light scattered on the dust goes more in front of the sample. The detector sensed intensity of scattered light over time. If the intensity is changing slowly against initial conditions over time, the particles are large. This is described by correlation function, which compare intensity before and after measurement at that moment. From Einstein-Stokes equation is known, that Brownian motion for small particles is bigger than for larger one, so the correlation function is decreasing sooner and faster over time than in case of larger particles. The attention must be taken also to the fact that the size of particles is proportional to intensity of detected light to the power of six. For example there will be amount of particles of size 5 nm and same amount of particles of size 50 nm – the intensity of 50 nm particles will be much higher than in case of 5 nm particles. So it is possible that larger particle outshine the smaller one.

To summarize this: in measured suspension are particles of many sizes, which are detected by detector over time and then using the special software are from the data extracted each individual correlation function of individual particles.

I recorded raw data such as Z-average and polydispersity index. Z-average is the primary and most stable parameter produced by the technique. It is also known as the cumulants mean. Cumulants analysis is a simple method, which analyses the correlation function using the Z-average (a mean value for the size) and polydispersity index (a width parameter).

The total volume of sample for three-day experiment was same as in the case of disc centrifuge, but Zetasizer required 200 μL of sample per one test. I adjusted settings of Zetasizer before every measurement. Each NPs had its own refractive index: nanodiamond – 2.418; magnetite NPs – 2.420; nZVI – 2.7. Other settings were for all of NPs always the same. Absorption of NPs was set to 0.01; as dispersant was chosen water with temperature heated to 25 °C, viscosity equal to 0.8872 cP and refractive index equal to 1.33. Equilibration time was adjusted to 60s. I used disposable cuvette ZEN0040. The Angle detection was adjusted to 178° Backscatter (NIBS) and as analysis model was chosen general purpose with normal

resolution. Finally, I set measurement duration, which was equal to 10 runs, where 1 run took 5 s.

## 3.2 Algal culture

I used *Chlamydomonas reinhardtii* as model organism. Single-cell green alga *Chlamydomonas* sp. was obtained from the Institute of Hydrobiology (Biology Centre v.v.i., Czech Republic). The culture was cultivated in Guillard-Lorenzen medium (WC) in a light box with the temperature set at (22 ± 2) °C and under light/dark regime of 14 h/8 h. The culture was harvested in its exponential growth phase.

Furthermore I measured concentration of culture by spectrophotometric optical density (OD) with wavelength of 680 nm with usage of UV-Vis spectrophotometer (Hach Lange, Germany) to get information about their quantity (Myers, Curtis, Curtis 2013). The culture was always tested in its exponential phase of growth. Therefore, I waited up to 7 to 10 days and always checked OD until the stock culture was ready for further tests.

For preparation of stock culture, I used mix of vitamins (100 µL), medium WC (100 mL) and another stock culture of *Chlamydomonas reinhardtii* (5 mL). At first I measured concentration of algae from different stock by spectrophotometer. Thereafter I waited up to 7 to 10 days and checked OD until the stock culture was ready.

Afterwards, I prepared shake flasks for experiment. When the concentration of prepared stock of algae had higher OD then 0.12, I had to dilute it by equation:

$$c_1 V_1 = c_2 V_2$$

where,

$c_1$ – concentration of stock culture, $c_2$ – needed concentration of algae,

$V_2$ – total volume of flask, $V_1$ – volume of algae use for experiment

for example: $OD_{680} = c_1 = 0.243;\ V_1 = ?;\ c_2 = 0.1;\ V_2 = 30\ mL$

$$V_1 = \frac{0.1 * 30}{0.243} = 12.35\ mL$$

I added this volume of algae to each shake flask together with 17 mL of WC medium and 17 µL of vitamin mix.

## 3.3 Comparison of different methods for culture characterization

I compared different analytical methods for characterization of algal culture at the beginning. Afterwards I decided what methods were satisfying and what could be used in toxicity tests.

One of the methods was cell counting. I used the hemocytometer (counting chamber) for determination of cell number. I pipetted 10 μL of algal stock culture on both sides of the hemocytometer and then counted number of cells within grid on the hemocytometer via microscope (AxioImager fluorescence microscope, ZEISS, Germany). I had to dilute the culture ten times or more when the number of cells was too high.

Second tested method was analysis of efficiency of photosynthesis, particularly measurement of quantum yield of photosystem II ($\Phi_{II}$). Quantum yield provides precise estimate about actual capacity of photosystem II for photochemical process (Weis, Berry 1987). It is a number of moles processed by photosystem II per one mole of absorbed photons. I measured it by AquaPen-C AP-C 100 (PSI, Czech Republic), which was set up on red light (620 nm) to perform measurement of algal culture.

AquaPen measures $\Phi_{II}$ equal to:

$$\Phi_{\mathrm{II}} = \frac{F_{\mathrm{V}}}{F_{\mathrm{M}}} = \frac{F_{\mathrm{M}} - F_0}{F_{\mathrm{M}}}$$

in a dark-adapted sample, where $F_V$ is maximal variable fluorescence and $F_M$ is maximal fluorescence in dark-adapted state. $F_V$ is equal to:

$$F_{\mathrm{V}} = F_{\mathrm{M}} - F_0$$

where $F_M$ is maximal fluorescence intensity and $F_O$ is minimal fluorescence in dark-adapted state.

I used 2.2 mL of stock culture for each measurement. The time required for each measurement was around 40 to 60 s.

The last method, which I tested for characterization of algal culture, was the determination of the concentration of cells by measuring OD of the culture using UV-

Vis spectrophotometer (Hach Lange, Germany) with a set up wavelength to 680 nm. Spectrophotometer measurement is based on Beer-Lambert law, which is saying, that the absorbance is directly proportional to the concentration of the absorbing substance of the sample. Absorbance *A* is equal to:

$$A = -log\frac{I}{I_0}$$

where *I* is the intensity of light passes through the sample and $I_0$ is the initial light intensity. It could be also written as:

$$A = \varepsilon \bullet l \bullet c$$

where $\varepsilon$ is molar absorptivity coefficient [$M^{-1}*cm^{-1}$], *l* is the length of path [cm] and *c* is the concentration of sample [$mol*L^{-1}$].

OD is measured by absorbance *A* and thickness *L* of sample. Optical density of spectrophotometer is then equal to:

$$OD = \frac{A}{L}$$

If $\varepsilon$ and *l* is constant and OD is lower than 0.25, the *c* concentration of sample could be easily calculated due to a linear relation between the concentration of cells and OD.

## 3.4 Toxicity tests

I used following methods for toxicity assessments: i) Determination of efficiency of photosystem II, ii) Determination of the concentration of chlorophyll *a* and iii) Detection of damage of cell membranes.

Right before performing the test, I measured concentration of algae by spectrophotometric optical density (OD) with a wavelength equal to 680 nm. Concentration of algae was prepared same like in Chapter *3.2* Algal culture and always adjusted to be 0.1. Concentration of freshly prepared NPs was equal to 0.01 g/L, 0.05 g/L and 0.5 g/L and was prepared by same way as in Chapter *3.1* Nanoparticles and their characterization. Exposition time was set up to 0h, 2h, 24h and 48h.

### 3.4.1 Determination of efficiency of photosystem II

The principle of this test was described in Chapter 3.3 Comparison of different methods for culture characterization. I used for this test AquaPen-C AP-C 100 (PSI, Czech Republic) (Figure 3.5). Used volume of sample was 2,2 ml per each test.

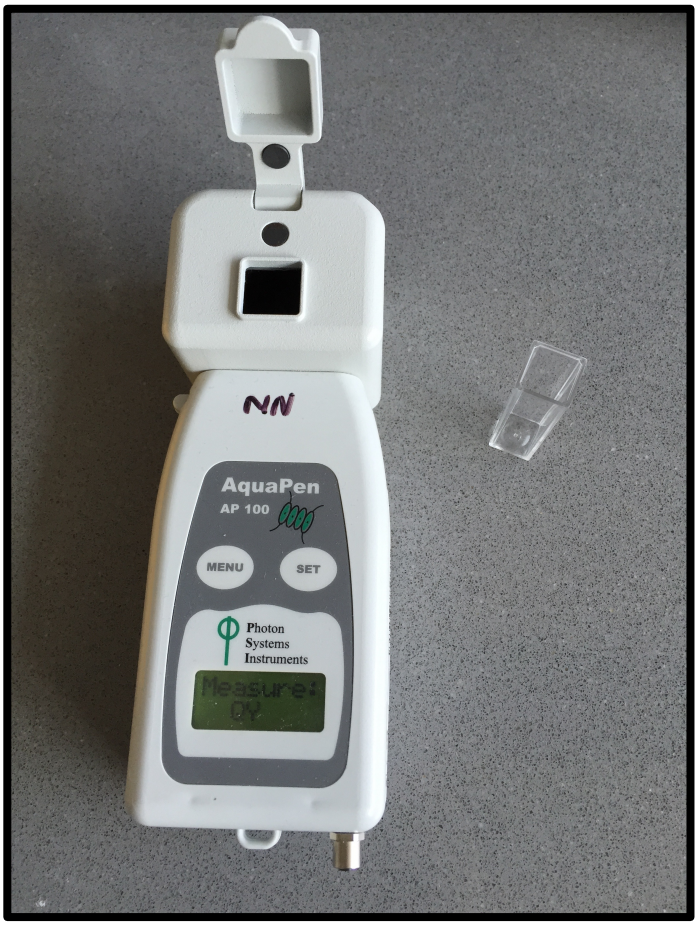


*Figure 3.5: On the left side Aquapen-C AP-C 100 (PSI, Czech Republic), on the right side cuvette*

### 3.4.2 Determination of the concentration of chlorophyll *a*

*Chlamydomonas reinhardtii* as green alga contains chloroplasts. This feature was used in determination of the concentration of chlorophyll *a*. I used for extraction of chlorophyll *a* combination of DMSO and acetone. I extracted 40 µL of algal stock culture (20 % of final volume) and added it into 160 µL (80 % of final volume) of a 1:1 mixture of DMSO and acetone (Mayer, Cuhel, Nyholm 1997). I followed this procedure for all samples at once. All samples were put on one 96-well plate. Thereafter I put it into dark place for 20 minutes. After this procedure, I measured the chlorophyll *a* fluorescence intensity by Synergy HTX Multi-mode reader (Bio-

tek, USA) (Figure 3.6) with setting: emission filter with wavelength of 645/40 nm, excitation filter with wavelength of 360/40 nm and sensitivity equal to 50 nm wavelength.

The principle of this method is based on chlorophyll *a* fluorescence. I used DMSO and acetone to obtain a stable and high fluorescence signal. Synergy HTX Multi-mode reader (Biotek, USA) was set to 360/40 nm wavelength to excite electrons in chlorophyll *a*. Chlorophyll *a* absorbed this energy and excited electrons. Part of excited electrons energy was emitted as fluorescence radiation with specific wavelength. This fluorescence radiation was measured using Multi-mode reader with filter of 645/45 nm. The numbers of algal cells were higher with higher fluorescence signal.

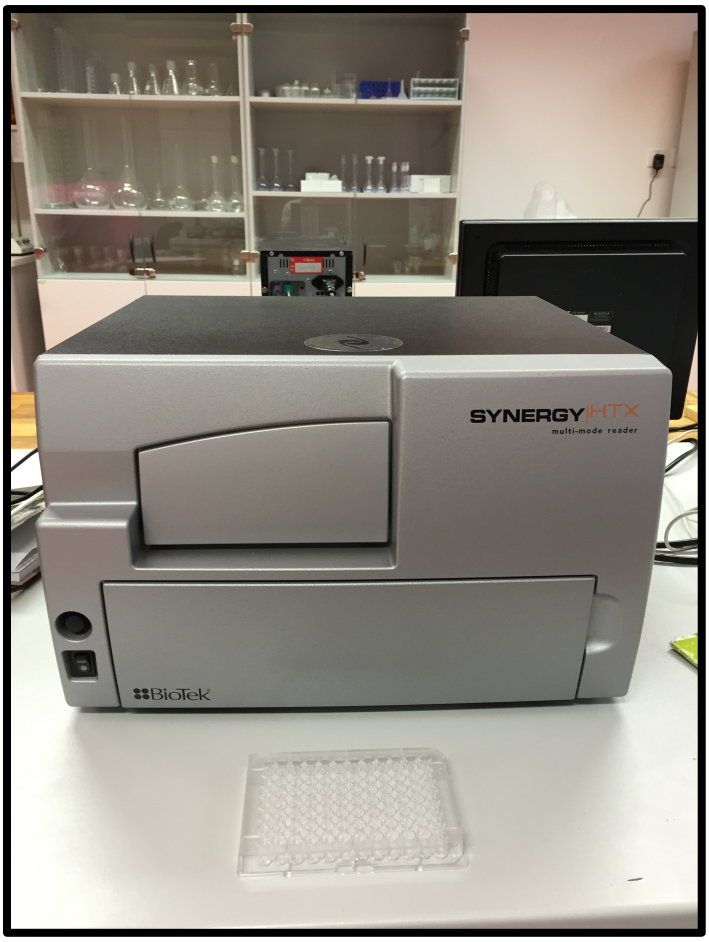


*Figure 3.6: Synergy HTX Multi-mode reader (Biotek, USA) and a 96-well plate*

### 3.4.3 Detection of damage of cell membranes

The damage of cell membranes was detected using fluorescence microscope (AxioImager, ZEISS, Germany)(Figure 3.7) and using SYTOX Green dye (Life Technologies, USA). I used 100 µL of algal sample per each test. I also prepared positive control – it was a control algal sample, which I put into heat treated for 15 minutes in order to permeabilized the plasma membrane. Afterwards, I pipetted samples into small tubes and added 2 µL of SYTOX Green. Thereafter I put them in dark place for 20 minutes. After this time I took 5 µL of sample and SYTOX Green mix from small tubes and put it on glass covered by a cover glass. I used two objectives on microscope, 09 AF 488 to see red autofluorescence of algae and 44 FITC to see green SYTOX Green dye.

The principle of this method was simple. If the algal cells were healthy and without damaged membranes, their colour was pure red and their shape was oval. If the algal cells had damaged membranes – the plasma membrane were permeabilized, the SYTOX Green dye leaked into the cells and stained DNA by green colour, visible in fluorescence microscope. These cells could be of red colour and oval too but with green spot inside. I took an image of cells and then calculated how many cells were damaged, i.e. labelled by SYTOX Green, and how many cells were healthy.

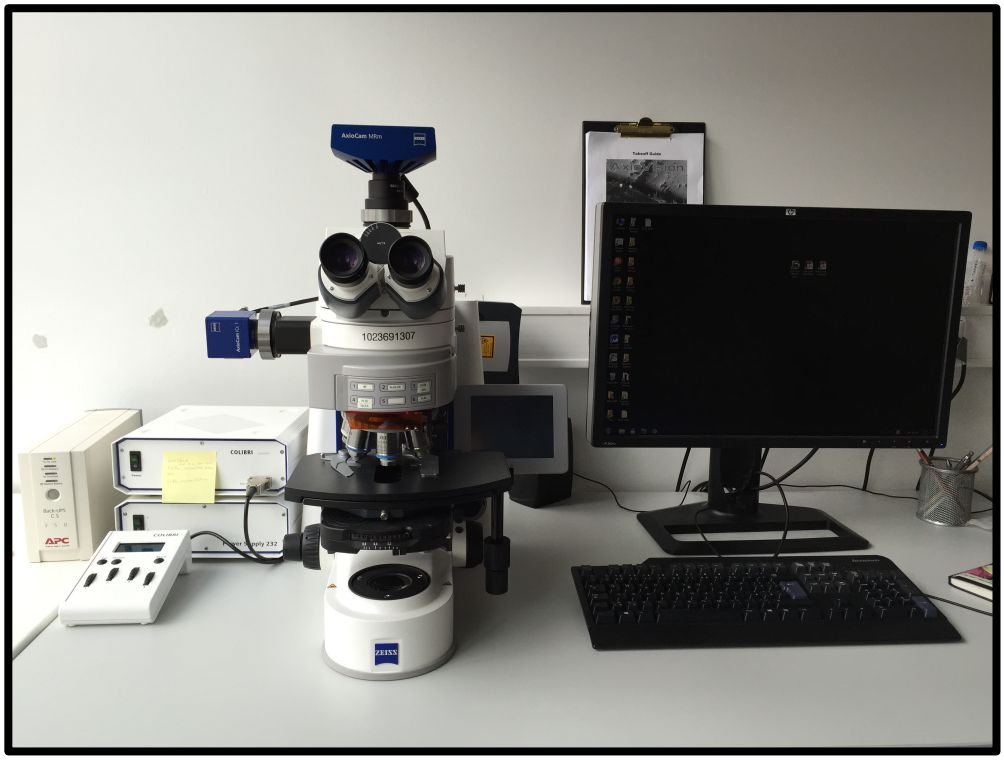


*Figure 3.7: AxioImager fluorescence microscope (ZEISS, Germany)*

## 3.5 Statistical analysis

I used for statistical analysis software GraphPad PRISM 6. Because the experimental data depend on two factors, time and concentration of NPs in solutions, I chose for analysis two-way (also called two-factors) ANOVA method. Repeated measures of two-way ANOVA were applied, because each row represented a different time point, so matched values were stacked into a subcolumn. Accordingly, the columns factor was named time and row factor concentration. Therefore each cell mean was compared with the negative control cell mean on that row. Dunnett multiple comparisons test was used to compute confidence interval and significance.

# 4 Results and discussion

In following chapters are summarized results from all experiments sorted by their type. All experiments were performed carefully to be repeatable and each treatment was done in triplicate to ensure quality data for statistical analysis.

Characterization of NPs was performed in two environments using the DCS and DLS methods. Furthermore several methods for characterization of alga *Chlamydomonas reinhardtii* were compared. According to the results it was then decided what methods will be used in toxicity tests.

Main aim of the toxicity test was comparison of three different methods: Determination of efficiency of photosystem II, Detection of damage of cell membrane and Determination of the concentrations of chlorophyll *a*, for detection of toxicity of nZVI, nanodiamond and magnetite nanoparticles using model organism – alga *Chlamydomonas reinhardtii*.

## 4.1 Characterization of NPs

I used for characterization disc centrifuge and Zetasizer. Results are shown in Appendix A (disc centrifuge) and Appendix B (Zetasizer). The recorded data are shown in several graphs. All graphs contain standard deviation, which were calculated from triplicate samples. For each method was recorded polydispersity index (a parameter of width), which is shown next to each NPs result. To understand the data: if the polydispersity index is high (in case of Zetasizer more than 0.7), the sample has a very broad size distribution.

According to the results in Appendix A, it could be seen, that disc centrifuge had problems with a lowest concentration 0.01 g/L and sometimes with 0.05 g/L. Results considerably fluctuated. In accordance, the polydispersity index showed broad distribution and even for nanodiamond in environment of WC medium it was not measurable at time point 48 h. In my opinion, the recorded data from the sample with higher concentration was more precise. Due to this, I summarized the size of each kind of NPs only for concentration of 0.5 g/L.

Appendix A shows that the change of environment has a minimal effect on process of aggregation of the NPs. Each kind of NPs has similar size in both DI and WC me-

dium. It seems, that the time had no effect on NPs too. Mean weight size of each kind of NPs (0.5 g/L) summarizes Table 4.1.

*Table 4.1: Mean weight particle size distribution in different environment and NPs using the disc centrifuge*

| NPs | Environment | Mean weight size [nm] |
|---|---|---|
| Nanodiamond | DI water | (560 ± 7) |
| | WC medium | (560 ± 30) |
| Magnetite | DI water | (650 ± 20) |
| | WC medium | (670 ± 40) |
| nZVI | DI water | (810 ± 70) |
| | WC medium | (880 ± 30) |

On the other hand, Appendix B is showing different data. Data measured by Zetasizer are shown in Table 4.2. I again compared the results for highest concentration of NPs (0.5 g/L) in different environments. Due to a polydispersity index these results had more limited distribution, but almost in all cases the largest aggregates of NPs.

*Table 4.2: Z-average size distribution in different environments and NPs using the Zetasizer*

| NPs | Environment | Z-average size [nm] |
|---|---|---|
| Nanodiamond | DI water | (490 ± 20) |
| | WC medium | (2200 ± 800) |
| Magnetite | DI water | (1120 ± 40) |
| | WC medium | (1600 ± 140) |
| nZVI | DI water | (490 ± 20) |
| | WC medium | (2100 ± 800) |

The values fluctuated and were not as precise as values from the disc centrifuge measurements. This could be because of wrong setup of duration time of measurement. Only 10 runs, where each run lasts 5 seconds, could be not enough for analysing these samples. The standard procedure was speeded up, because of time-consuming measurements and high occupancy of Zetasizer.
An advantage was that Zetasizer measures triplicates at once, so it was more comfortable and less time consuming than disc centrifuge.

I tried to find any information about used NPs from the companies that produced them. However, International Technology Center from which were nanodiamonds

obtained was the only company, that has published some information about them (ITC 2013). According to them the average primary particle size was 4 nm and the agglomerate size in water suspensions 200 nm. None of these information were confirmed by disc centrifuge or by Zetasizer. This is very common finding that information on size from manufacturer differs from the actual NPs size (Phenrat et al. 2007; MacCuspie et al. 2011)

The important information are: NPs generally aggregate similarly in both environments and the time has no effect on the process. More precise values gave DCS analysis.

## 4.2 Comparison of different methods for culture characterization

First method was direct cell counting using the optical microscope. This method is recommendable due to a small volume of used sample. The time required for the count depends on the number of cells. However, according to the data shown in Figure 4.1 it is obvious that this method was not satisfying for further usage in toxicity tests. There were large error bars and the growth curve was not fully correct. The errors could be explained by a human factor, counting of the cells probably requires more experienced person. This problem could be easily overcome with long-term praxis, but for my work it was found as not suitable due to limited time.

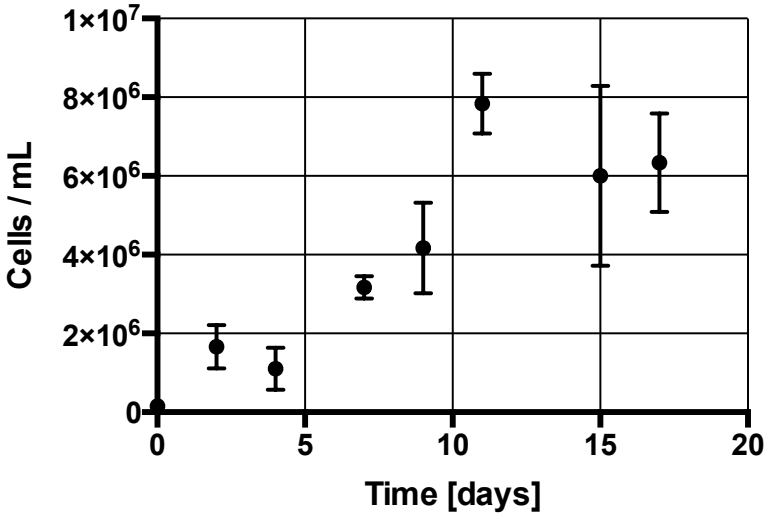


*Figure 4.1: Cell counting method for analysis the concentration of C. reinhardtii over time*

Second method tested for characterization of algal culture, was the measurement of efficiency of photosynthesis, concretely quantum yield of photosystem II ($\Phi_{II}$). In a principle with higher $\Phi_{II}$ values were the number of algal cells higher too. In Figure 4.2 are clearly seen a large error bars and unexpected shape of growth curve in the first few days of the test. This could be caused by my initial inexperience with this method; other authors had no problem with accurate $\Phi_{II}$ measurement (Vandamme et al. 2012; Pan et al. 2011). This method was found to be suitable for characterization of status of algal culture during further toxicity tests, because it is easy method and requires minimum time.

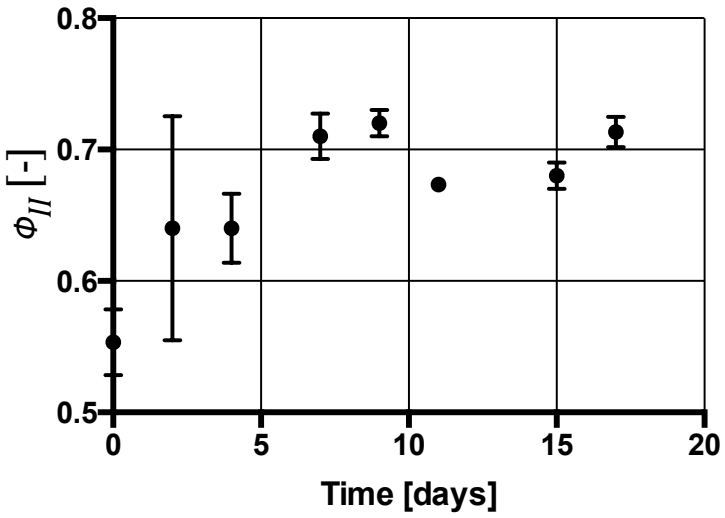


*Figure 4.2: Efficiency of photosystem II of C. reinhardtii measured by $\Phi_{II}$ over time, $\Phi_{II}$ is dimensionless quantity*

Last method was the measurement of cells concentration using spectrophotometer. The values of OD were based on exponential growth of cells and increasing turbidity of sample. This method was used as control of growing algal culture, because is fast and cheap, but the numbers were indicative only. It was not suitable for toxicity tests, because I used NPs with properties such as dark colour, enormous surface and possibility of occurrence of aggregates, which could increase the turbidity of sample and make impossible to measure correct values of cells concentrations.

Figure 4.3 is clearly showing, that the stock culture of alga was healthy and in growth phase during the experiment. According the increasing values of OD is obvious, that the number of cells was increasing over time too.

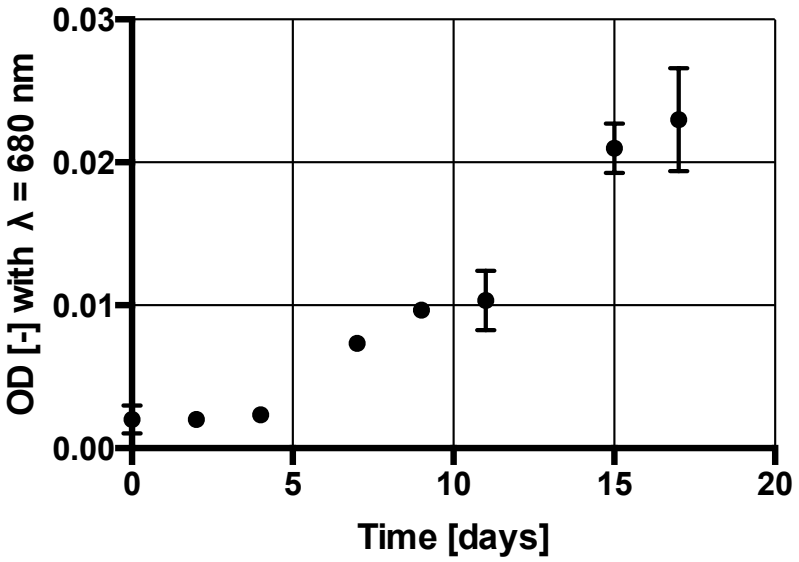


*Figure 4.3: Determination of the concentration of cells measured by spectrophotometer over time, OD is dimensionless quantity*

The experiences and information, which I obtained from these methods, I used in toxicity assessments. Some of these methods were promising and I used them with upgrades in toxicity tests, some were, after discussion, evaluated as unsuitable.

## 4.3 Toxicity tests and statistical analysis

First method was determination of efficiency of photosystem II using the quantum yield ($\Phi_{II}$), which I measured by Aquapen-C AP-C 100 (PSI, Czech Republic).

Columns in few graphs (Figure 4.5, Figure 4.6, Figure 4.7, Figure 4.9) were labelled with mark [*], which point at significantly different results from the negative control sample. In the legends could be seen the concentrations of each NPs along with the interpreted colour.

According to the Figure 4.4 it could be seen, that the alga was photosynthesizing well over time and exposition to different concentrations of magnetite NPs had not a great influence on the $\Phi_{II}$.

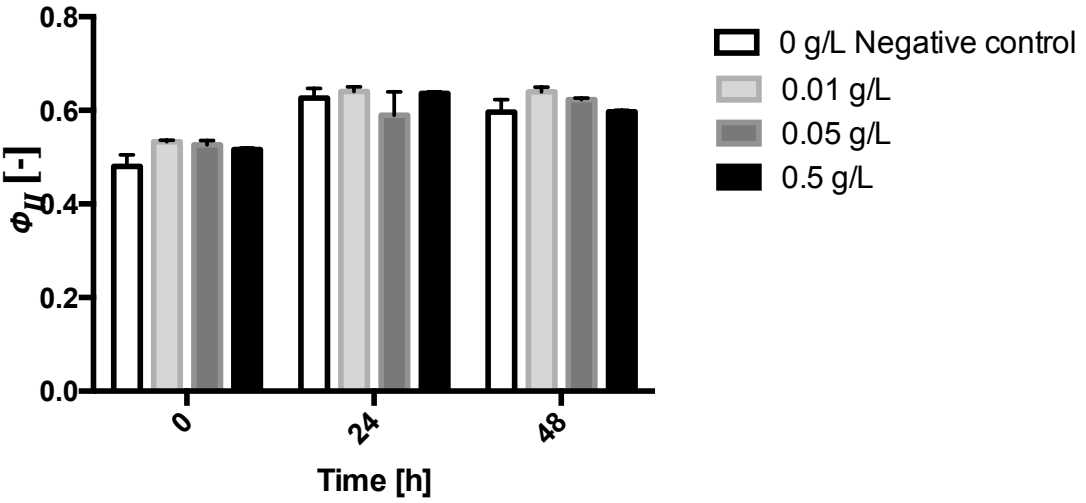


*Figure 4.4: Analysis of magnetite NPs toxic effects on efficiency of photosystem II of C. reinhardtii over time using $\Phi_{II}$*

The same trend of influence by nanodiamond is shown in Figure 4.5, despite the fact, that at time 0 h and 24 h are due to statistic analysis significant differences.

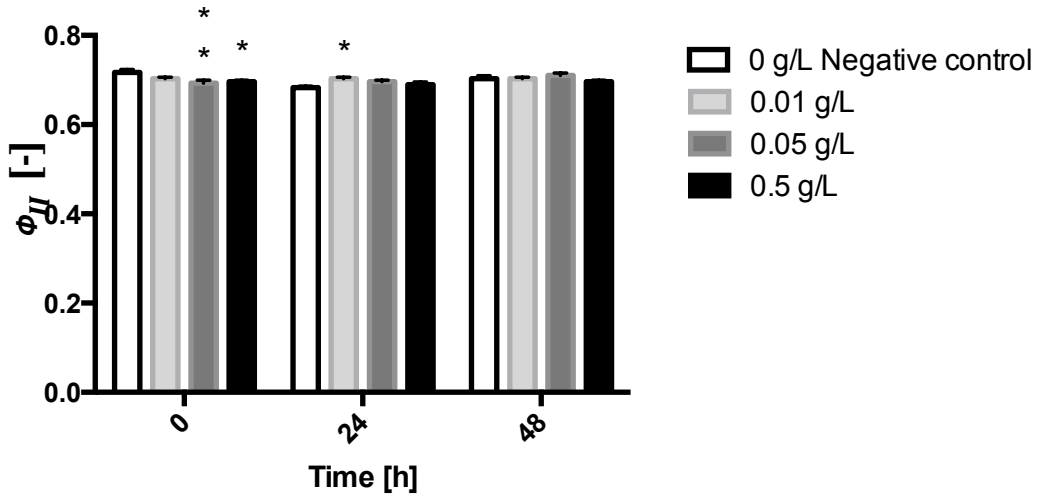


*Figure 4.5: Analysis of nanodiamond toxic effects on efficiency of photosystem II of C. reinhardtii. over time using $\Phi_{II}$, Significance level: *P <0.05; **P <0.01*

The significant difference means that the labelled [*] concentrations (Figure 4.5) had in this specific time higher influence on alga than other. It is calculated from the mean difference of negative control and the specific NPs concentration.

If the nanodiamond had a higher influence on $\Phi_{II}$, it should be best observed in the final time of experiment (48 h). In this case the values of $\Phi_{II}$ of each nanodiamond concentration were even more similar to negative control at the time 48 h than in

case of magnetite NPs. In conformity with this fact, it is obvious that nanodiamond had even smaller effect on $\Phi_{II}$ than magnetite NPs.

The results from the experiment with nZVI are shown in Figure 4.6.

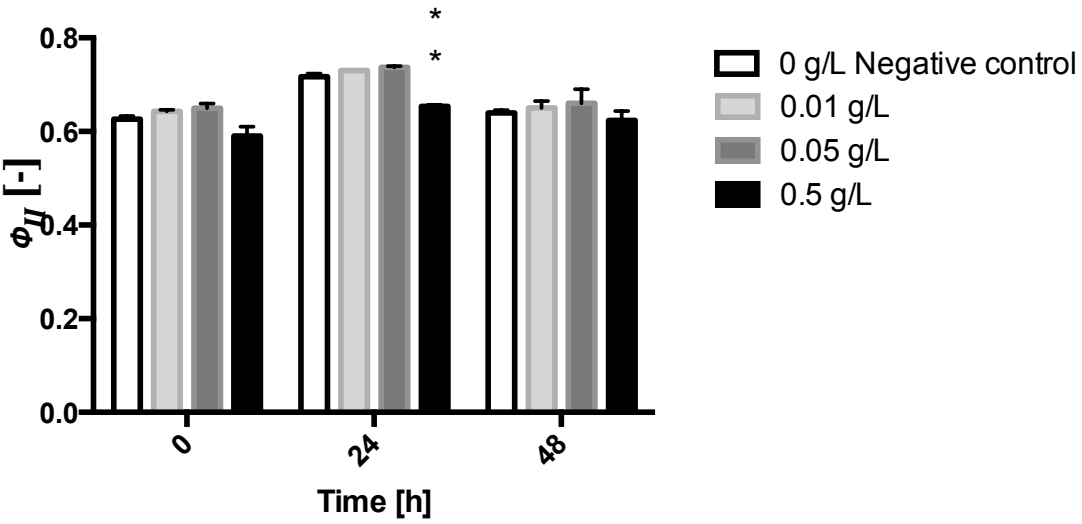


*Figure 4.6: Analysis of nZVI toxic effects on efficiency of photosystem II of C. reinhardtii. over time using $\Phi_{II}$, Significance level: **P <0.01*

The results of nVZI were a bit unexpected in comparison with results of magnetite NPs and nanodiamonds (Figure 4.6). Concretely, efficiency of photosystem II was higher in samples with nZVI than in controls without NPs. Despite small fluctuations the values were also consistent with the rest of the results.

This method showed no significant trend of effect of NPs on $\Phi_{II}$. It was not very suitable for this toxicity assessment, although the authors of similar research showed opposite (Perreault et al. 2010). They investigated toxicity of copper oxide NPs using chlorophyll *a* fluorescence imaging in *Lemna gibba* and found out that the quantum yield of photosystem II decreased with higher concentration of NPs. The differences in results could be explained by the fact, that neither nZVI nor magnetite and nanodiamond are high toxicants. According to this fact the determination of efficiency of photosystem II might be not enough precise for such a measurement.

This method was the least informative in comparison with determination of the chlorophyll *a* and detection of damage of cell membranes. On the other hand it is the fastest method among the others. Therefore, it is good for quick analysis of

pure algal culture, but for more advanced measurement of effect of NPs I would recommend to choose different method.

The second method was determination of concentration of chlorophyll *a* by Synergy HTX Multi-mode reader (Biotek, USA). Figure 4.7 shows low influence of magnetite NPs on the fluorescence signal of the extracted chlorophyll *a*.

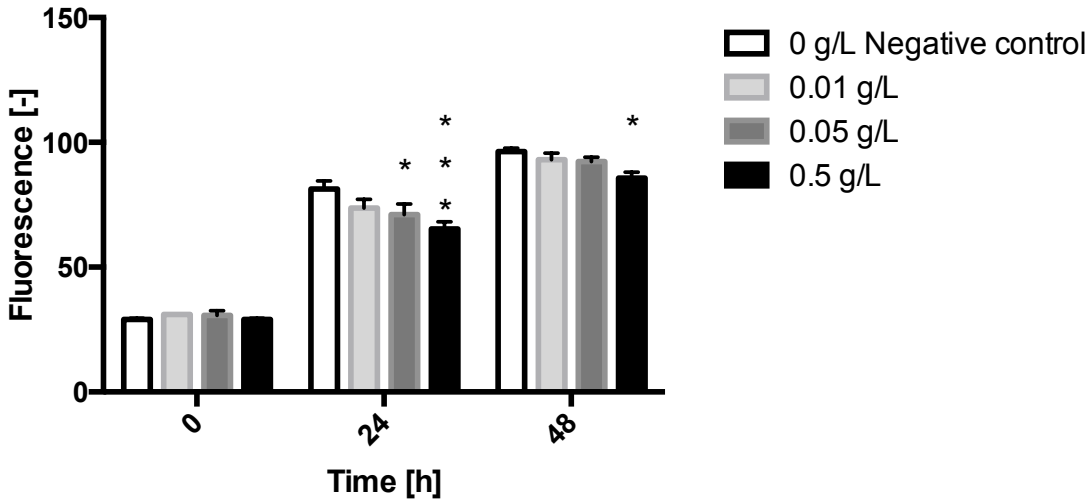


*Figure 4.7: Analysis of toxic effects of magnetite NPs on the chlorophyll a of C. reinhardtii over time using the fluorescence signal measured by the Multi-mode reader, fluorescence is dimensionless quantity, Significance level: ***P <0.001; *P <0.05*

The fluorescence signal was almost the same for all samples at time 0 h, but with increasing time there was a trend. It seems that with increasing concentration of magnetite NPs, is the fluorescence, i.e. the concentration of chlorophyll *a*, decreasing. This is most evident after 24 h of exposition, where the concentration of 0.5 g/L had high significant difference in comparison with the negative control. The culture could somehow recover after 48 h of NPs exposition and, therefore the effect was less significant.

However, the results of nanodiamond shown in Figure 4.8 were less informative. The values of fluorescence more varied than in the case of magnetite NPs. Despite the results, the trend is probably the same, although not significant. Higher concentration of NPs causes lower concentration of chlorophyll *a*.

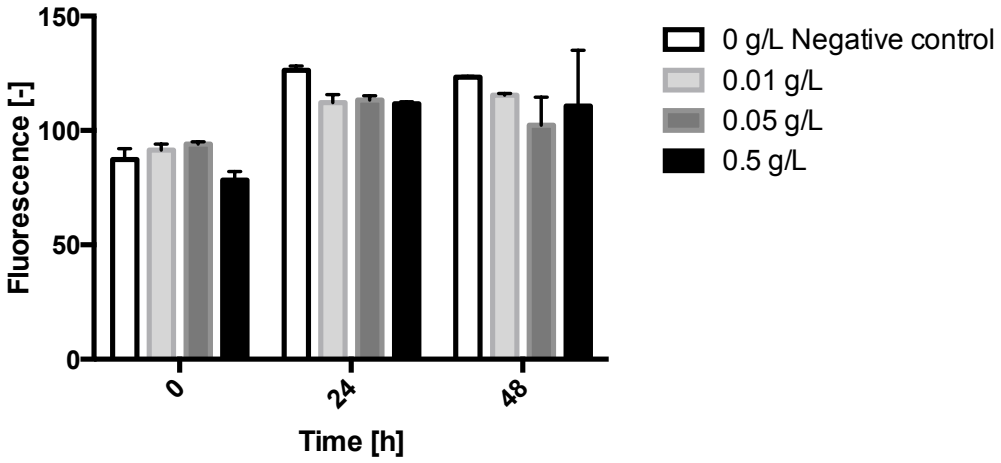


*Figure 4.8: Analysis of toxic effects of nanodiamond on the chlorophyll a of C. reinhardtii over time using the fluorescence signal measured by the Multi-mode reader*

It is interesting that all fluorescence values in Figure 4.8 are much higher than in case of Figure 4.7. In my opinion there are two explanations. First, it is because of different growth phase of alga. Even though I always adjusted the concentration of alga to 0.1, the original stock might be older and the condition of alga different. Second, nanodiamond bright fluorescence at 600 nm to 800 nm (Chapter 2.3 Areas of applications of NPs) and due to this fact the fluorescence signal is higher (Xiao et al. 2015).

Nevertheless, the most important for toxicity assessment is always comparison of NP-treated samples with negative control without NPs.

In the case of testing nZVI the trend of increasing effect with increasing NP concentration is even more obvious (Figure 4.9). The highest concentration of NPs has the highest influence on the fluorescence signal of extracted chlorophyll *a*, although other nZVI concentrations did not show significant effect.

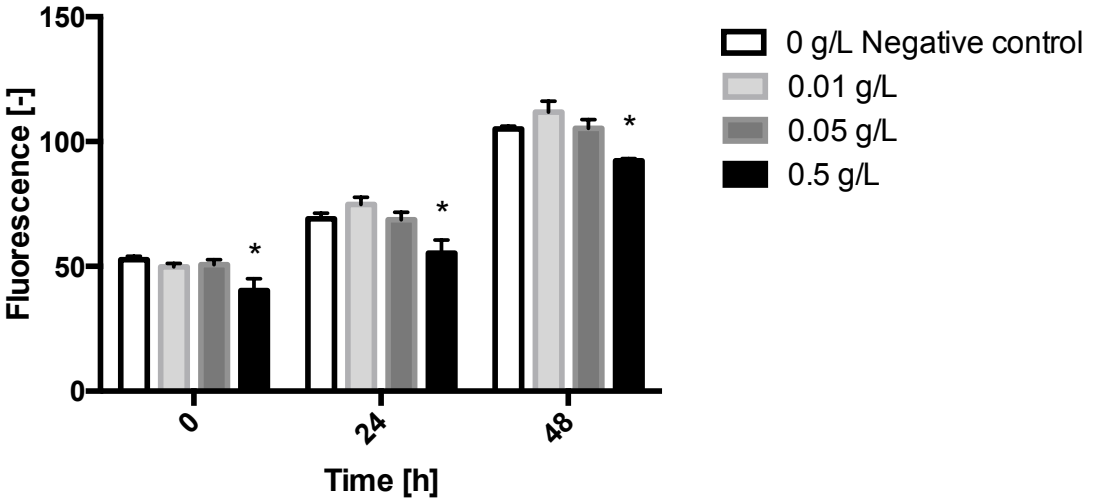


*Figure 4.9: Analysis of toxic effects of nZVI on the chlorophyll a of C. reinhardtii over time using the fluorescence signal measured by the Multi-mode reader, Significance level: *P <0.05*

This trend is similar to the trend in article by (Mayer, Cuhel, Nyholm 1997), where the condition was alike in this experiment. They find out that with increasing concentration of toxicant is the fluorescence of extracted chlorophyll *a* decreasing. This could confirm the hypothesis about the trend and the accuracy of the results. According to the results is this method more precise and informative than the determination of efficiency of photosystem II. However, it is more time consuming and for precise results is more advanced and expensive fluorometer necessity.

The last method, with which I tested toxicity of the NPs, was the detection of damage of cell membranes using the fluorescence microscope. All graphs show the percentage of unaffected cells after exposition with NPs (Figure 4.10, Figure 4.12, Figure 4.15). Unaffected cells had healthy membranes that did not allow SYTOX Green dye to leak into the cell and stain the DNA. In the experiment I prepared triplicate of one treatment on glass slide and then I calculated how many cells were labelled or not. These data were recorded and thereafter converted to percentage, where 100% matched the value of negative control. Due to this procedure it was impossible to display the standard deviation on graphs in representative way. Nevertheless, each point represents an average of three samples.

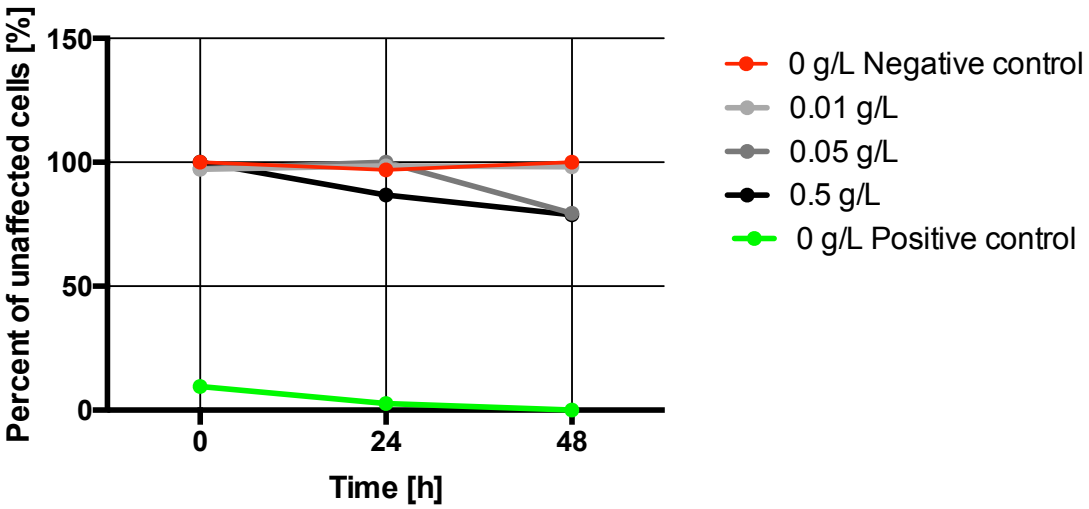


*Figure 4.10: Analysis of magnetite NPs influence on the cell membranes of C. reinhardtii over time using the SYTOX Green dye*

In Figure 4.10 there are results of effect of magnetite NPs on cell membranes. Positive control was made to test proper action of SYTOX Green dye (Figure 4.11).

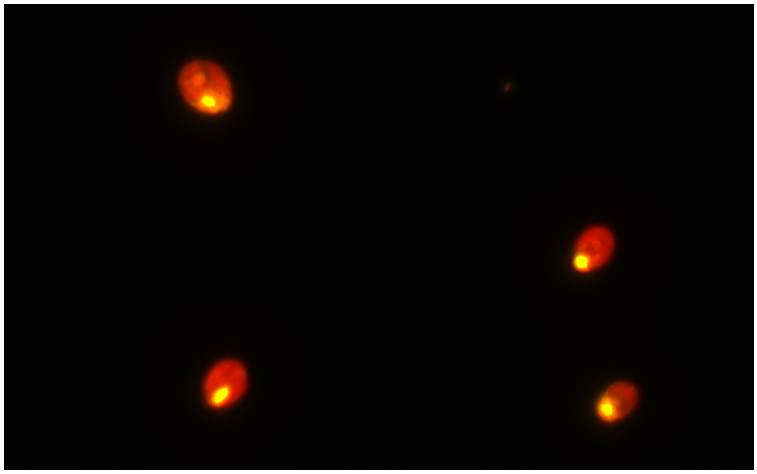

*Figure 4.11: Demonstration of the SYTOX Green dye on the positive control sample of C. reinhardtii*

If all membranes of cells were somehow damaged, all cells were labelled. Due to this, Figure 4.10 clearly shows that SYTOX Green dye worked properly. Negative control was the right opposite, should be 100 % in all times and according to results in Figure 4.10 it is true. Figure 4.10 also exhibits trend, which was gained using other methods with increasing concentration of NPs is the percentage of unaffected cells decreasing. These results are in agreement with research by (Machado, Soares 2012) who analysed toxicity of 1-pentanol on *Pseudokirchneriel-*

*la subcapitata* using the SYTOX Green dye. The authors found out that the percentage of cells with permeabilized plasma membrane was increasing with increasing concentration of toxicant. This means that, in this case magnetite NPs somehow disturbed and permeabilized the cell membranes, although not in extensive way.

The problem starts with other tested NPs. Figure 4.12 shows one of them. Negative control is lower than the highest concentration of nanodiamond after 48 h of incubation.

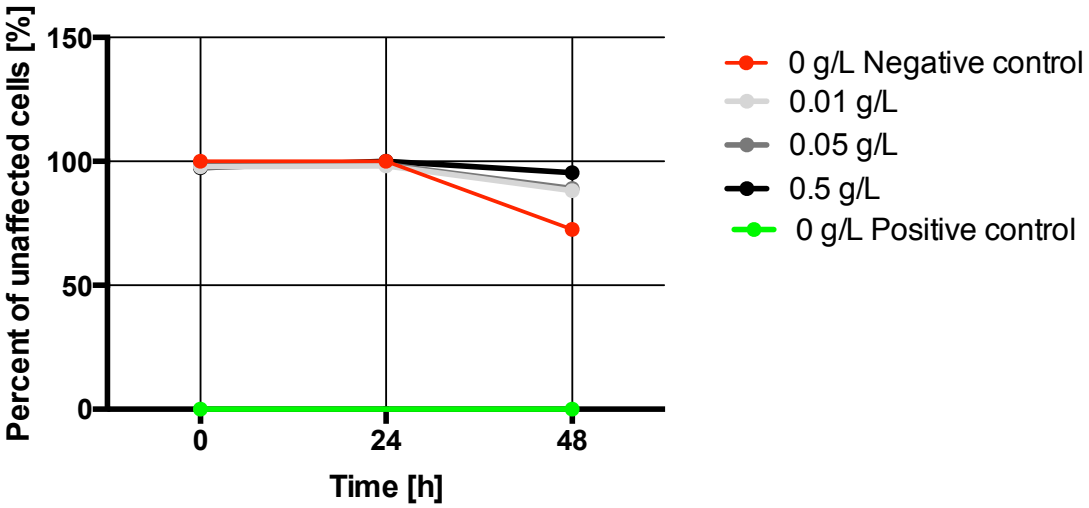


*Figure 4.12: Analysis of nanodiamond influence on the cell membranes of C. reinhardtii over time using the SYTOX Green dye*

In this specific case, it is because of malfunction in the sample (Figure 4.13).

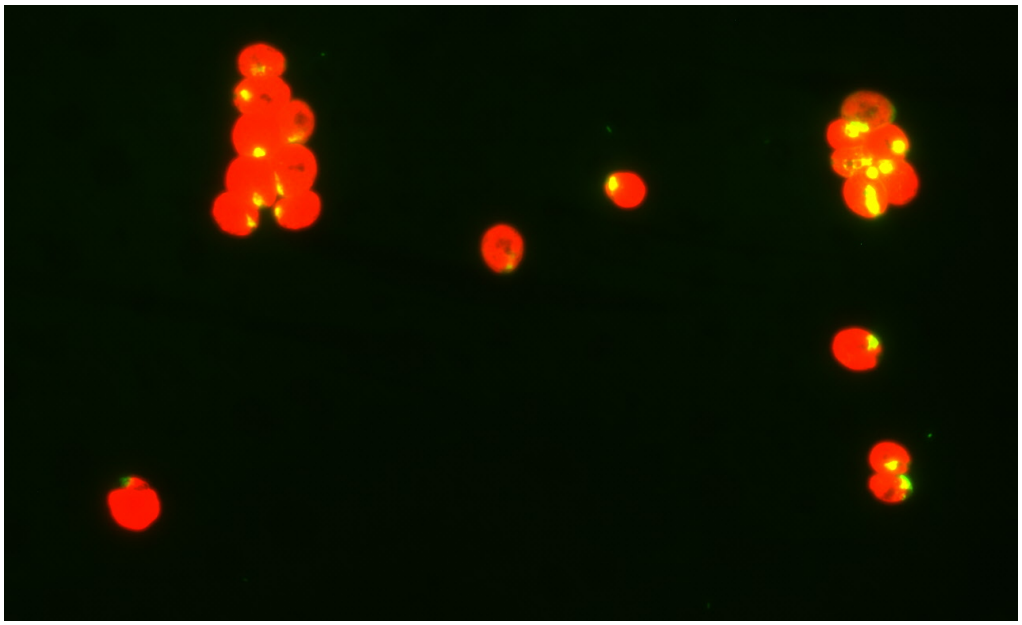

*Figure 4.13: Malfunction in negative control sample of analysis of nanodiamond influence on the cell membranes of C. reinhardtii. after 48 h of incubation*

All cells should be unstained and only red colour as it is shown in Figure 4.14. However, as shows the values of positive control, SYTOX Green dye worked properly.

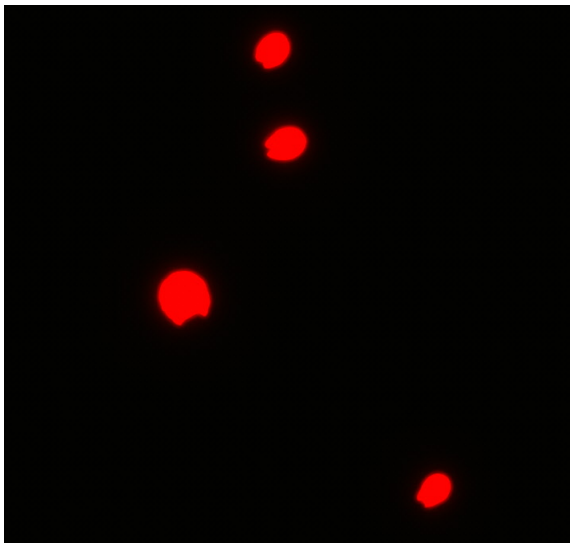

*Figure 4.14: Living cells without disrupted cell membrane*

Another problem is shown in Figure 4.15, where is not obtained any trend. This could be explained by two reasons. First, nZVI did not disturb the plasma membrane and cells were unaffected in all concentrations, second, the measurement was inaccurate because of similar reason as in a case of nanodiamond (Figure 4.12).

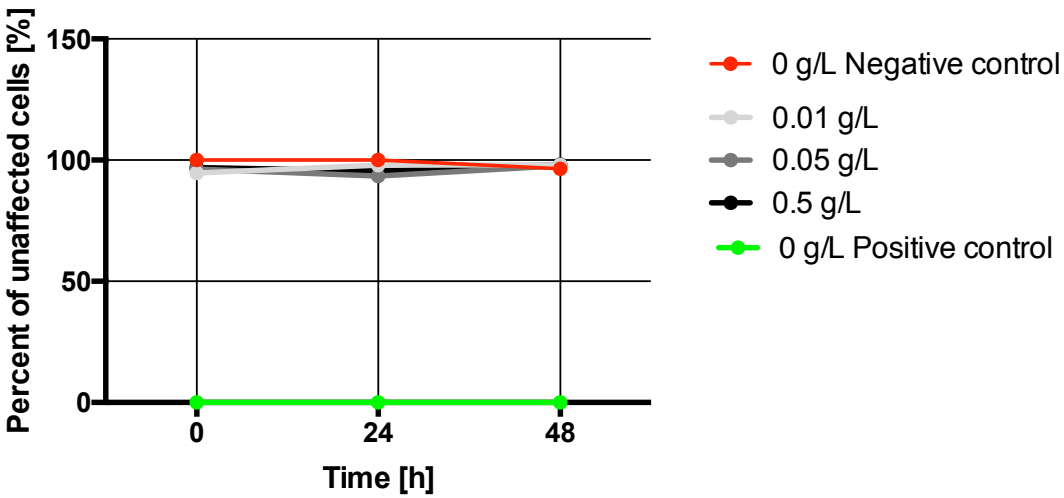


*Figure 4.15: Analysis of nZVI influence on the cell membranes of C. reinhardtii over time using the SYTOX Green dye*

This method has some disadvantages – the data fluctuated and results were not so solid as with determination of chlorophyll *a*. Errors could be explained by human mistake with cell calculations. In my opinion, this method is not very suitable for accurate analysis, but it is splendid for observation of algae and NPs. Using this method, it is possible to see what is exactly happening with sample in precise time. This attribute is without a doubt an advantage.

Comparing all three methods, it is necessary to realize, what is important and what the main task of the study is. Whether it is easy feasibility, duration, accuracy or ability to see what is exactly happening with tested organism in the sample. Each method has a part of these attributes.

If we want cheap and quick information on how the cells grow and how the culture is healthy, the determination of efficiency of photosystem II using $\Phi_{II}$, is the best choice.

If precision is requested, I would recommend the determination of concentration of chlorophyll *a*.

And, finally, if we want to see how NPs aggregate, interact with cells or just have "own eyes" evidence of what is exactly happening with the sample, the detection of damage of cell membranes using the fluorescence microscope is the best choice.

In my opinion the best method, based on my experiences with this comparison, is the determination of chlorophyll *a*. It is precise, fairly fast and solid stable results giving method, which is (with expectation of quality fluorometer) cheap and consumes less volume of sample than other two methods. After all with this method I obtained the best results at all, which were also confirmed by independent research (Mayer, Cuhel, Nyholm 1997).

# 5 Conclusion

This bachelor thesis provides the elementary knowledge on comparison of three analytical methods for nanotoxicity assessment, describing their advantages and disadvantages together with recommendation of their usage based on the results. Furthermore thesis provides helpful advices in using the correct statistical analysis and in using the DLS and DCS methods for the NPs characterization. All methods were discussed together with the results in Chapter 4 Results and discussion.

In my opinion, this thesis has achieved outlined goals and brings at least a small contribution to research of toxicity of NPs. Because the field of nanotoxicology is still largely unexplored, I would love to continue and help to develop this discipline in my future studies.

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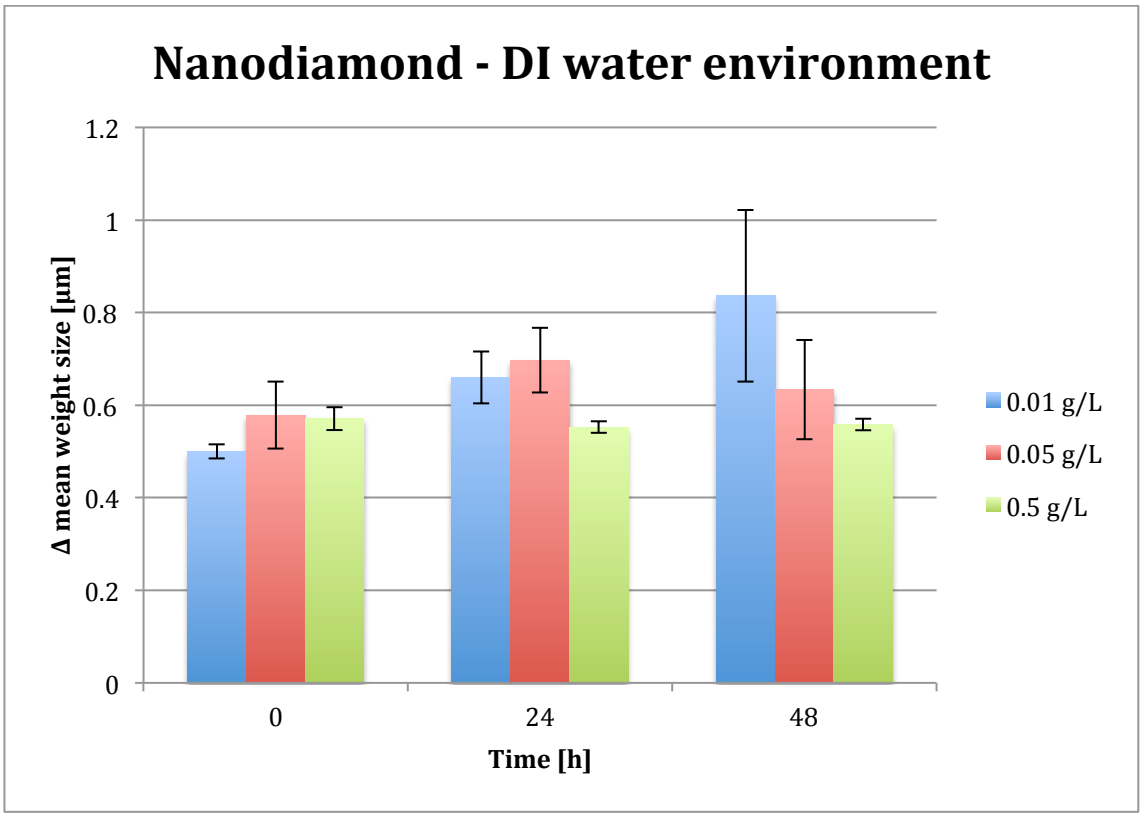
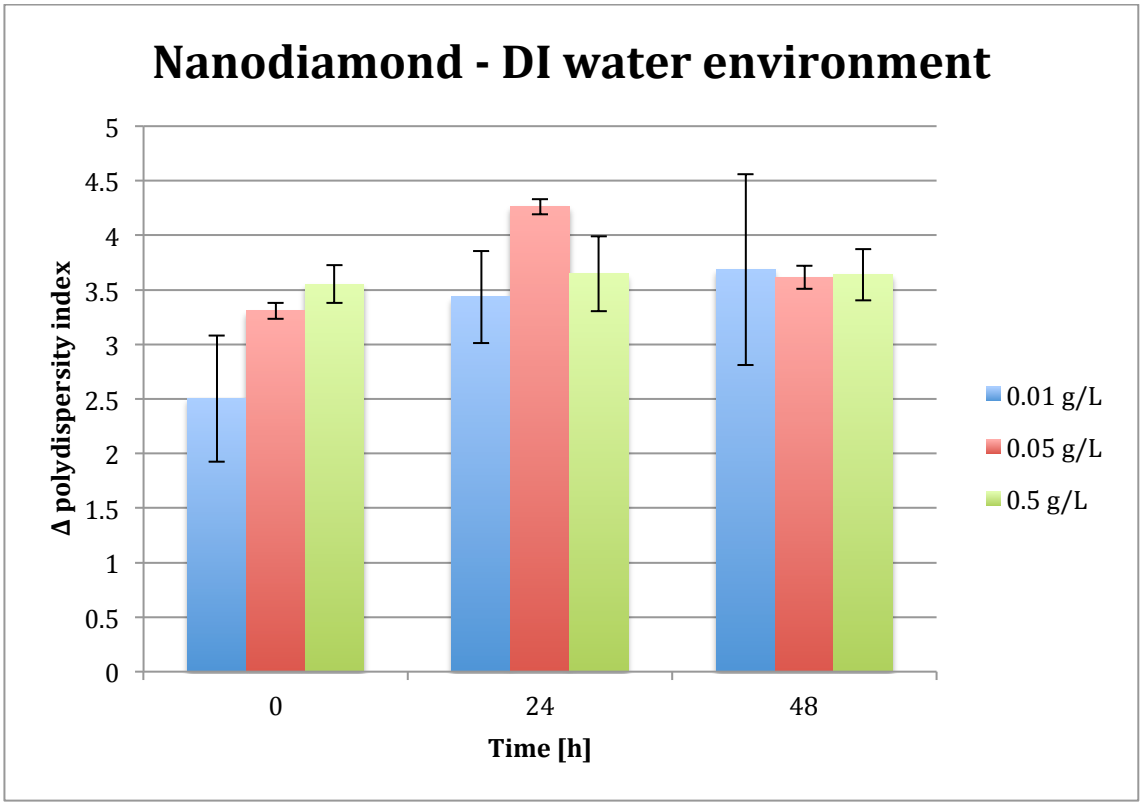
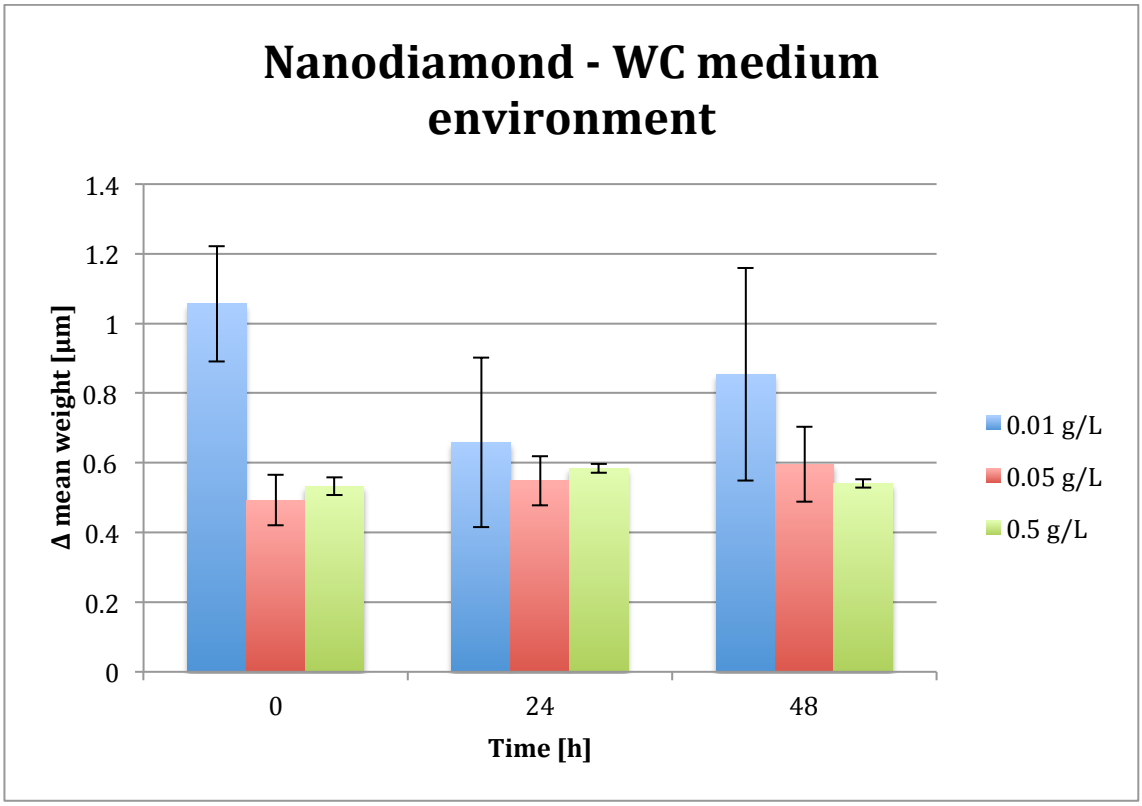
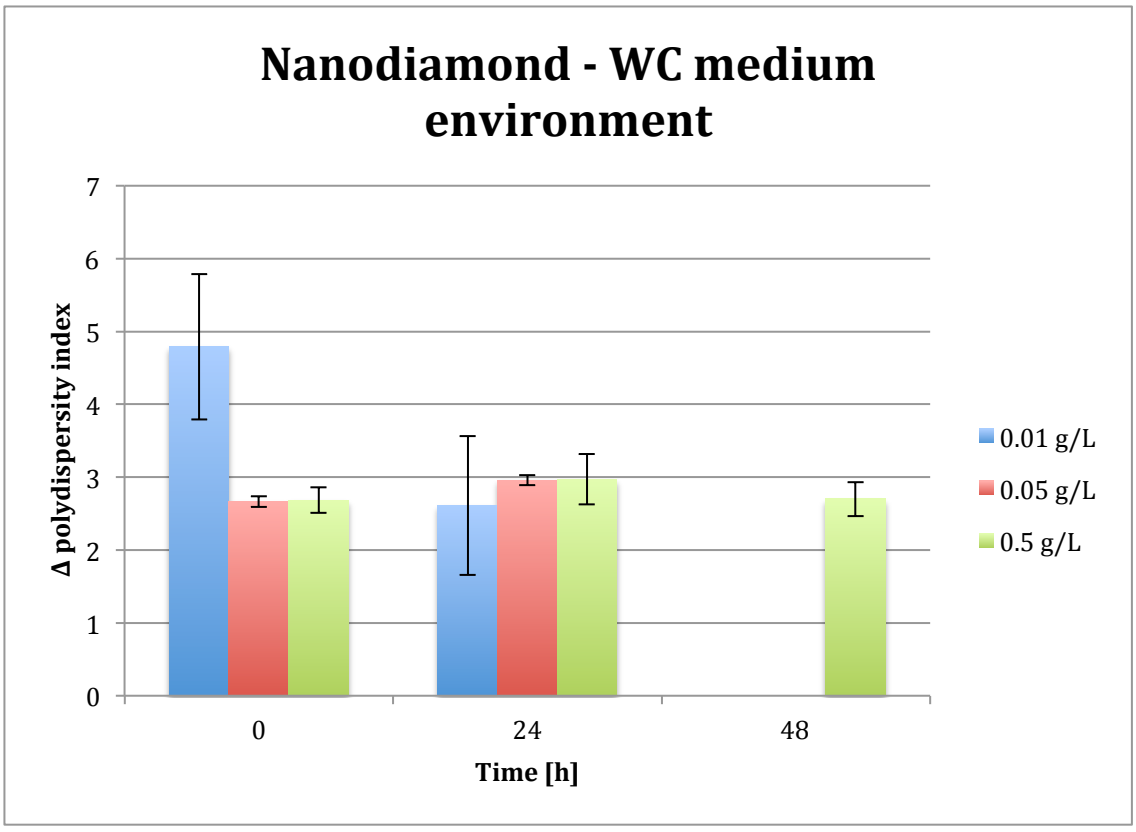
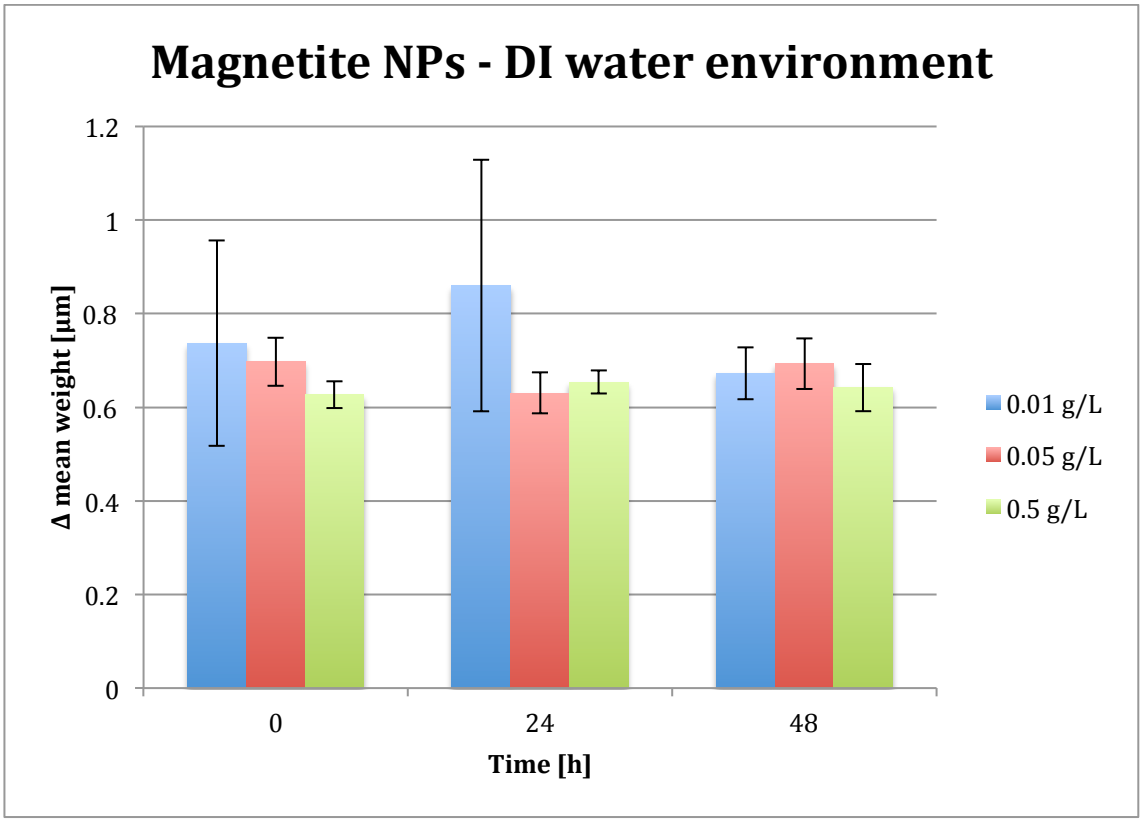
# Appendix A

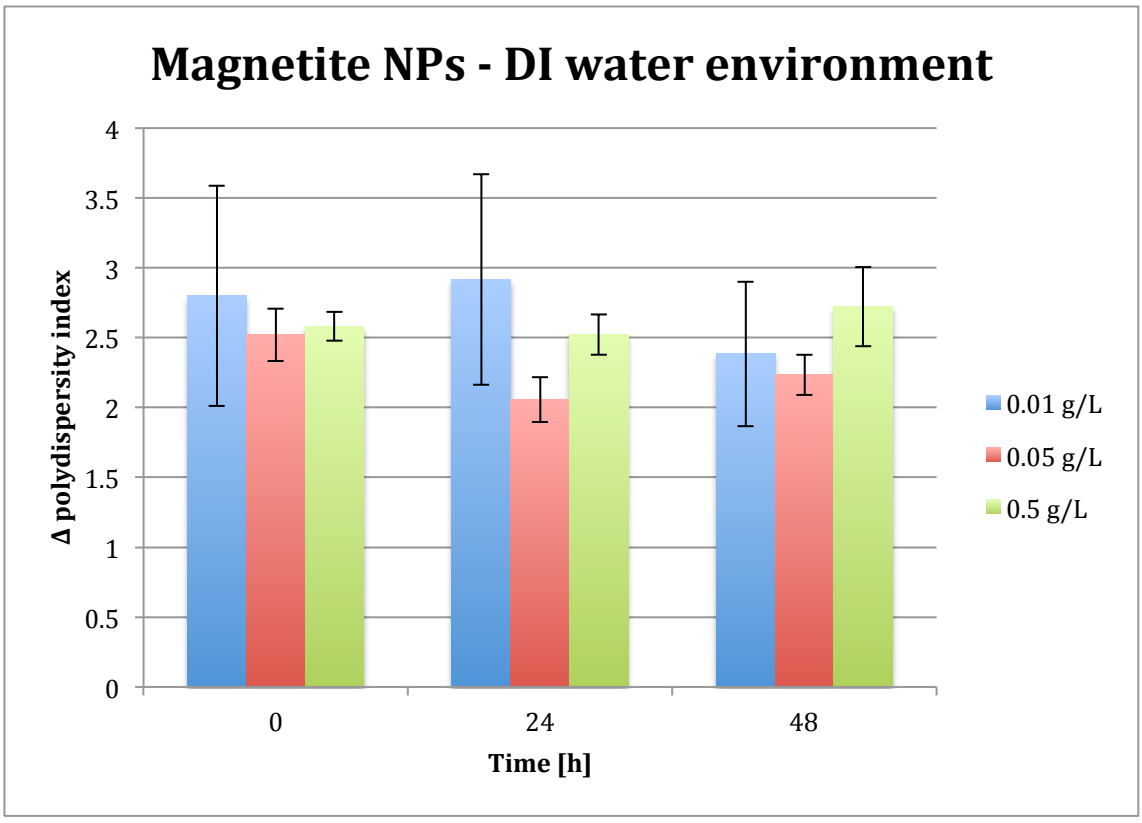

Nanodiamond - DI water environment
Δ mean weight size [µm]
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

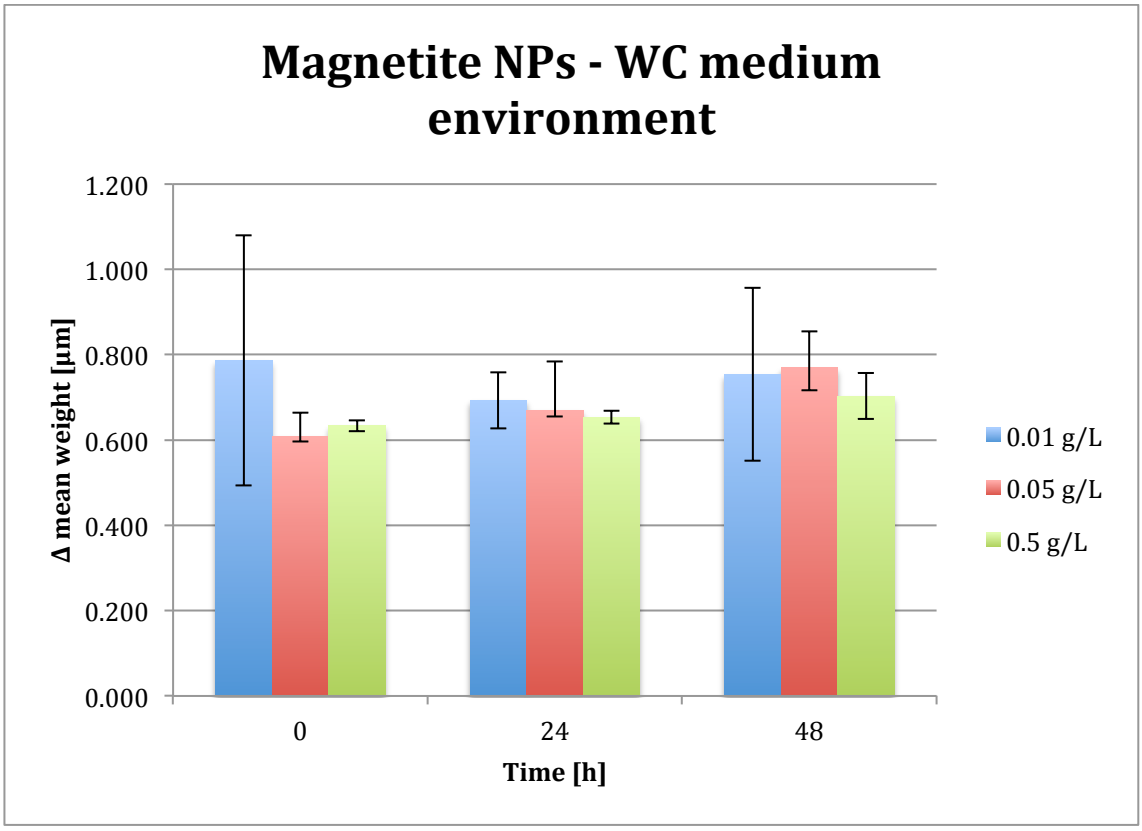

Nanodiamond - DI water environment
Δ polydispersity index
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

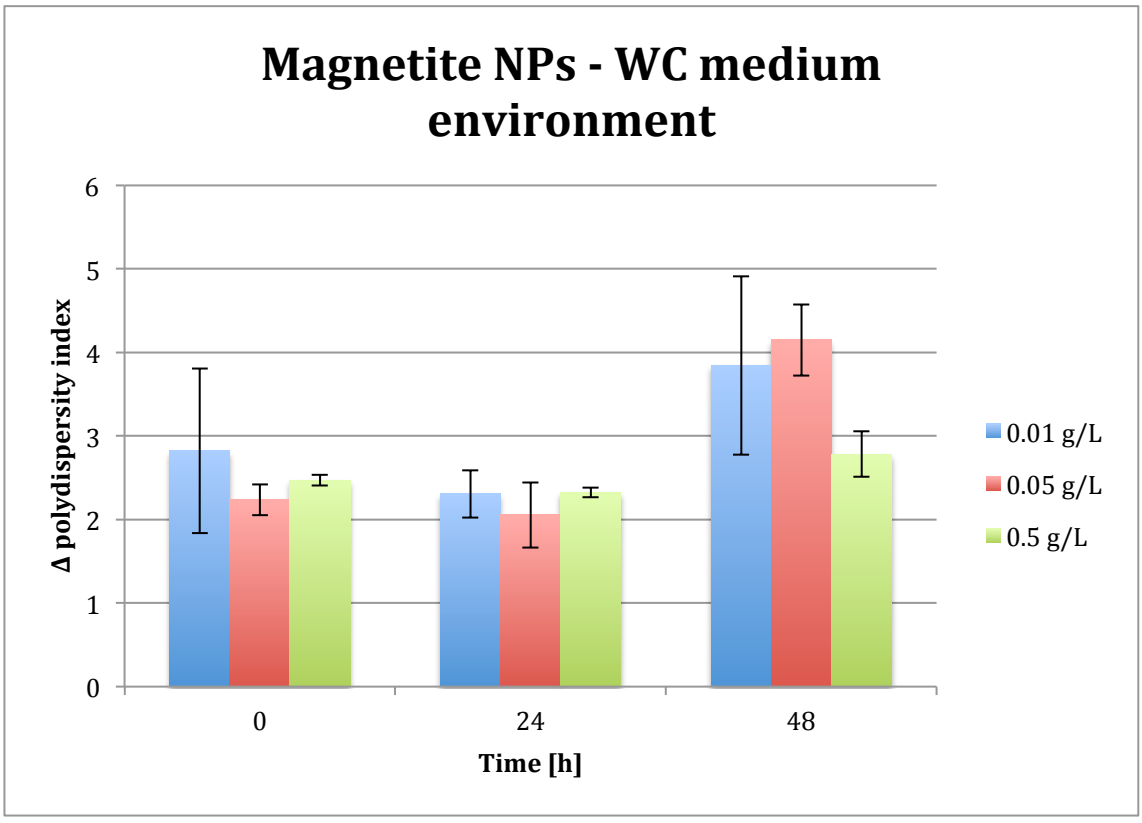

Nanodiamond - WC medium environment
Δ mean weight [µm]
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

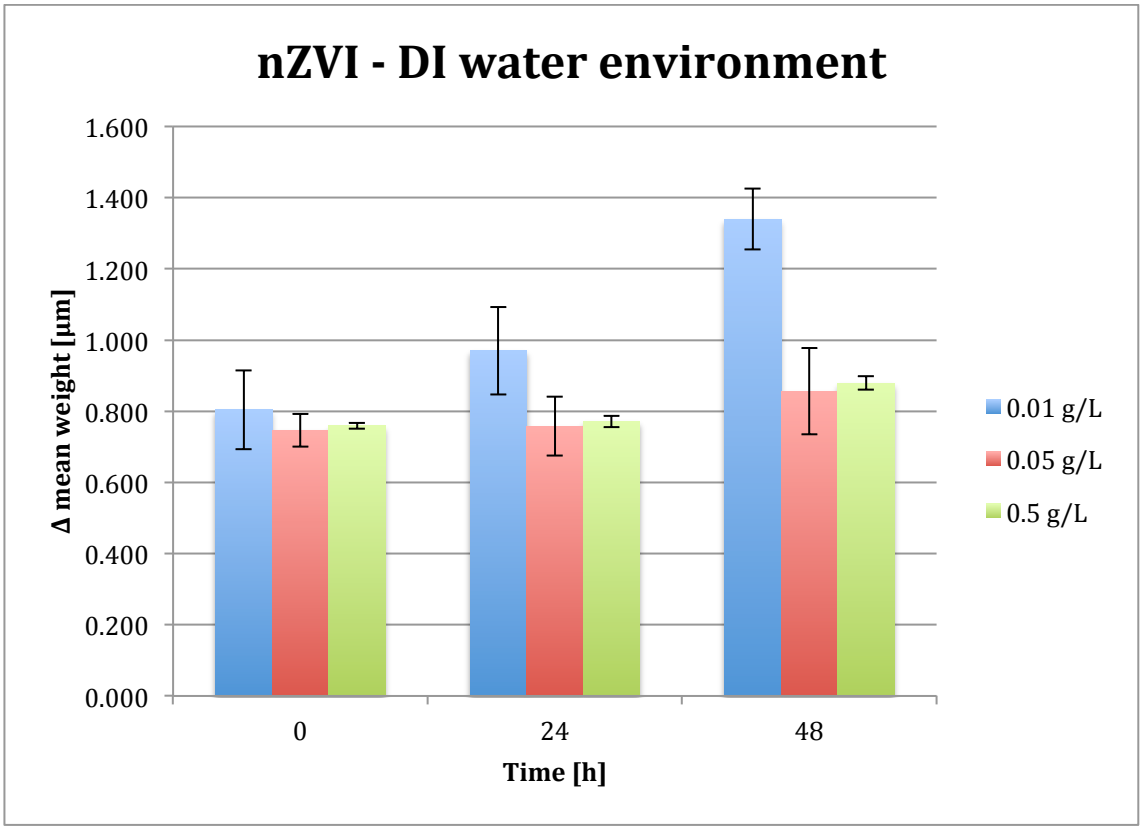

Nanodiamond - WC medium environment
Δ polydispersity index
7
6
5
4
3
2
1
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

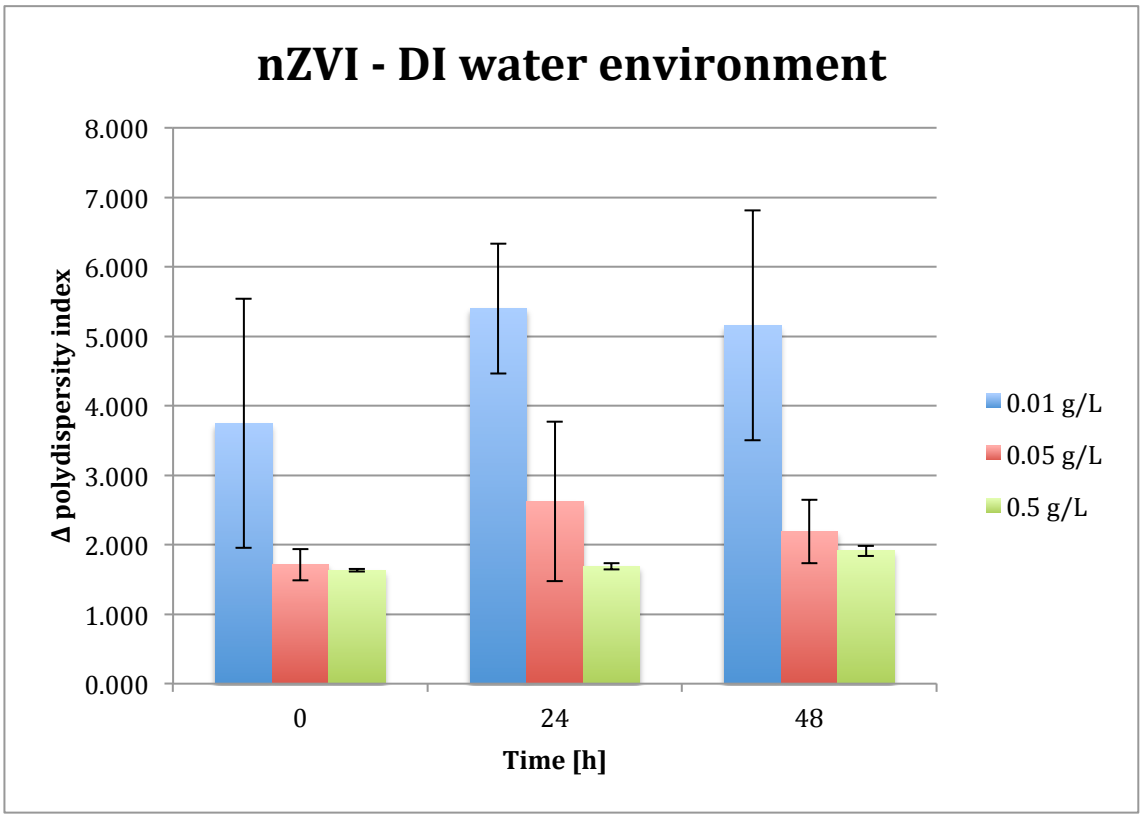

Magnetite NPs - DI water environment
Δ mean weight [µm]
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

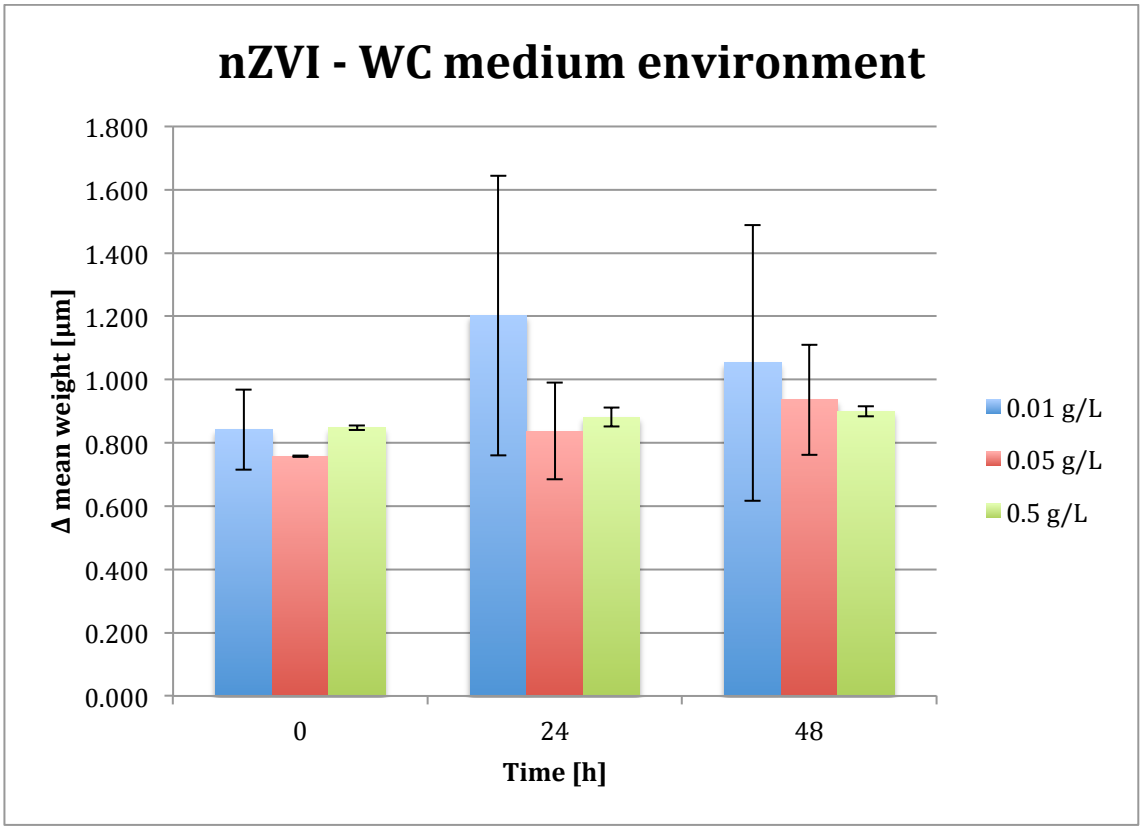

Magnetite NPs - DI water environment
Δ polydispersity index
4
3.5
3
2.5
2
1.5
1
0.5
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

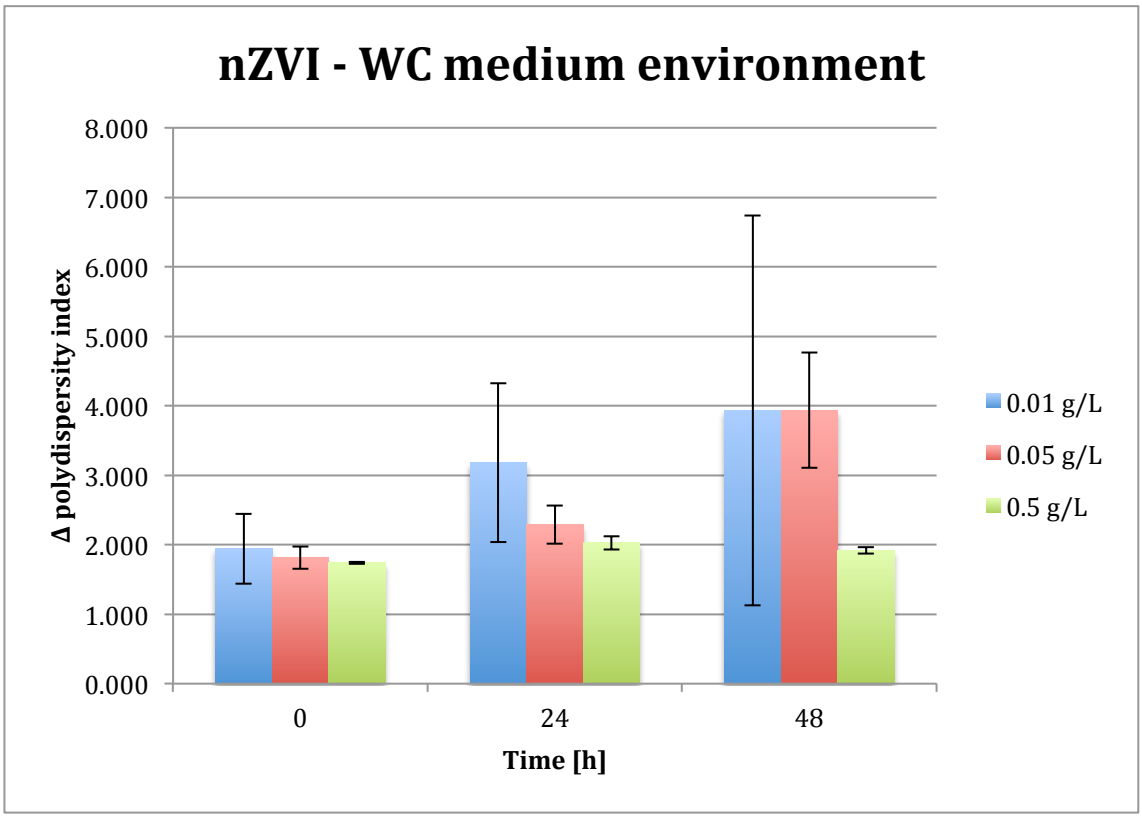

Magnetite NPs - WC medium environment
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Δ mean weight [µm]
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


Magnetite NPs - WC medium environment
6
5
4
3
2
1
0
Δ polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

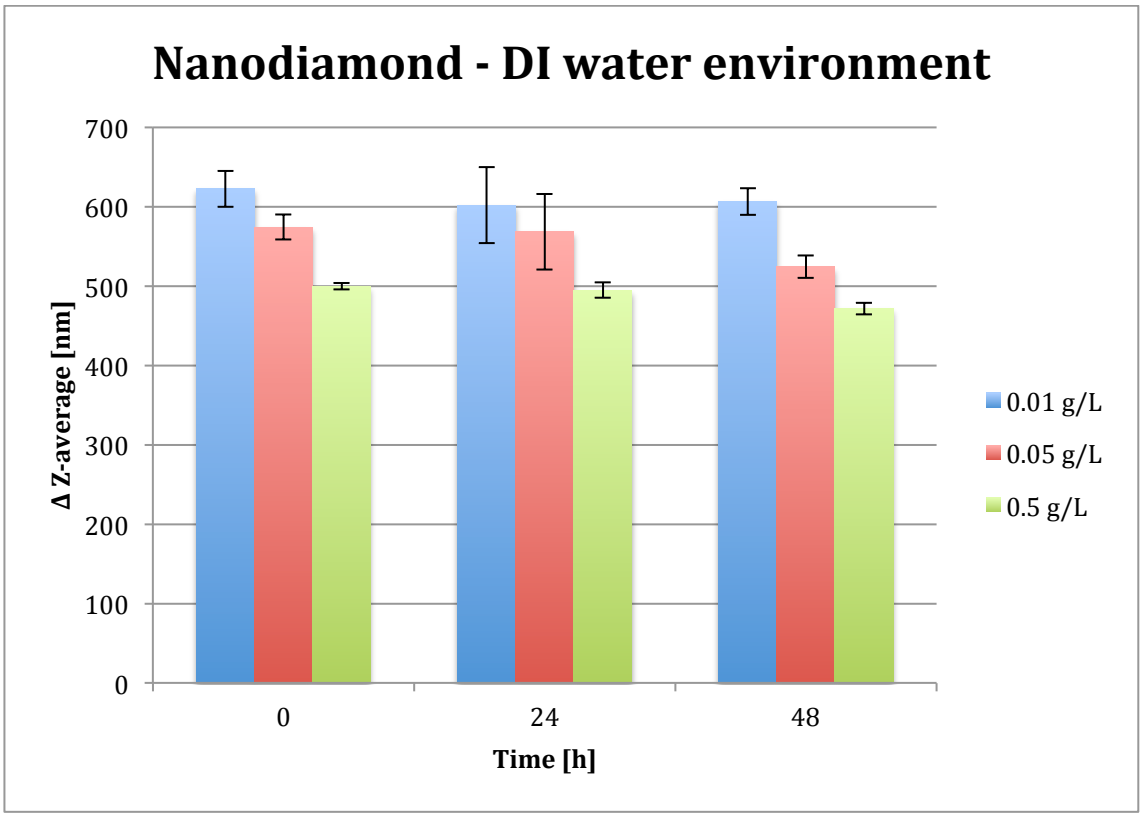

nZVI - DI water environment
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
Δ mean weight [µm]
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

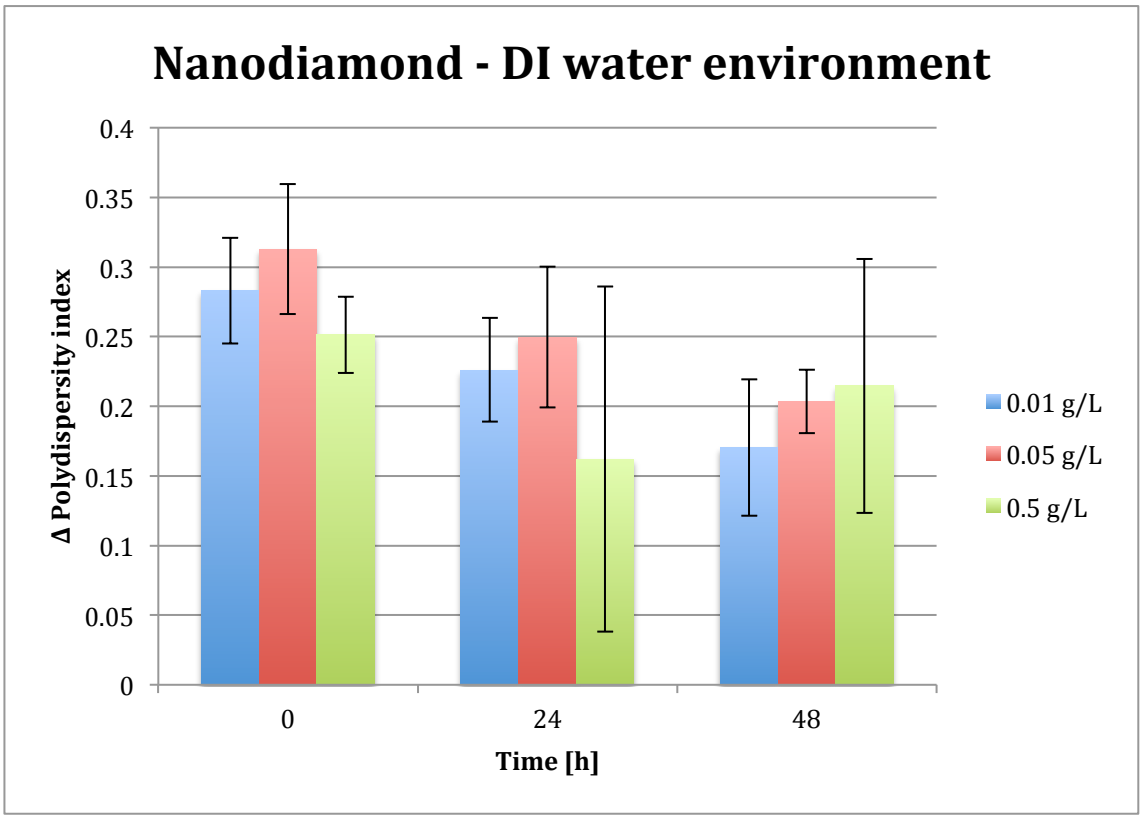

nZVI - DI water environment
8.000
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
Δ polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

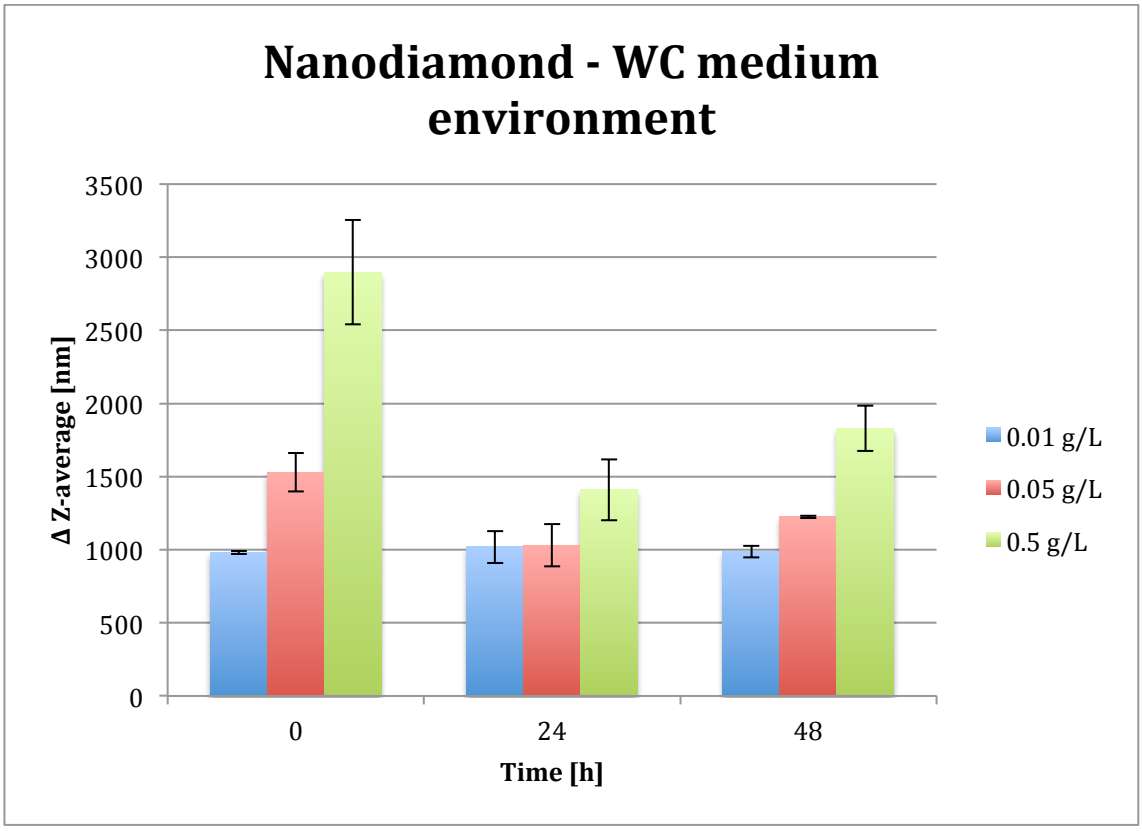

nZVI - WC medium environment
Δ mean weight [µm]
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

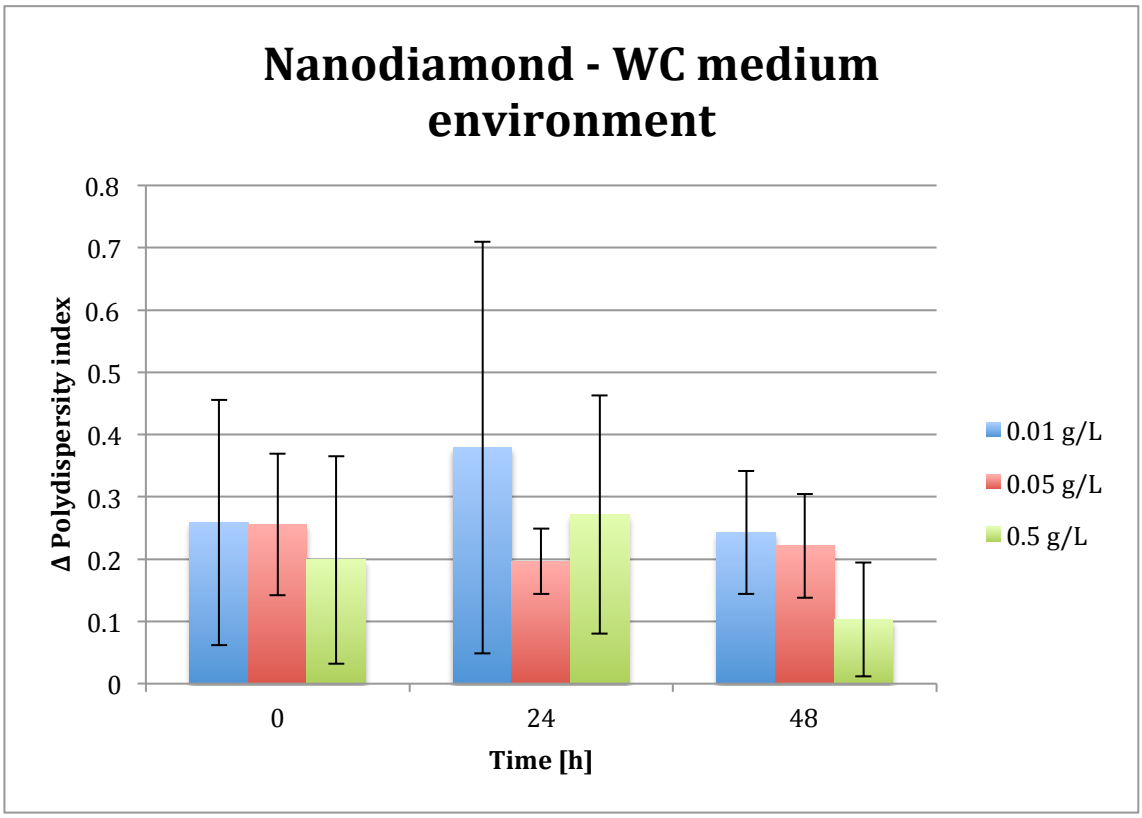

nZVI - WC medium environment
Δ polydispersity index
8.000
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

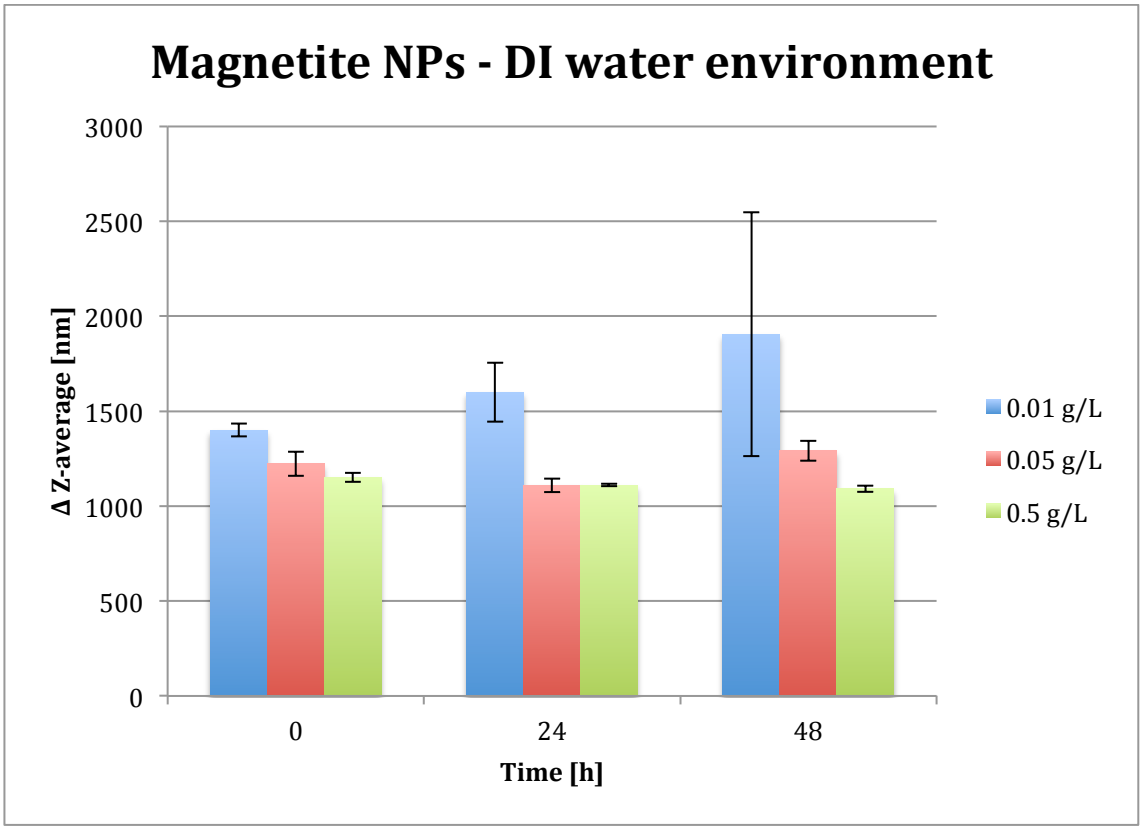
## Appendix B

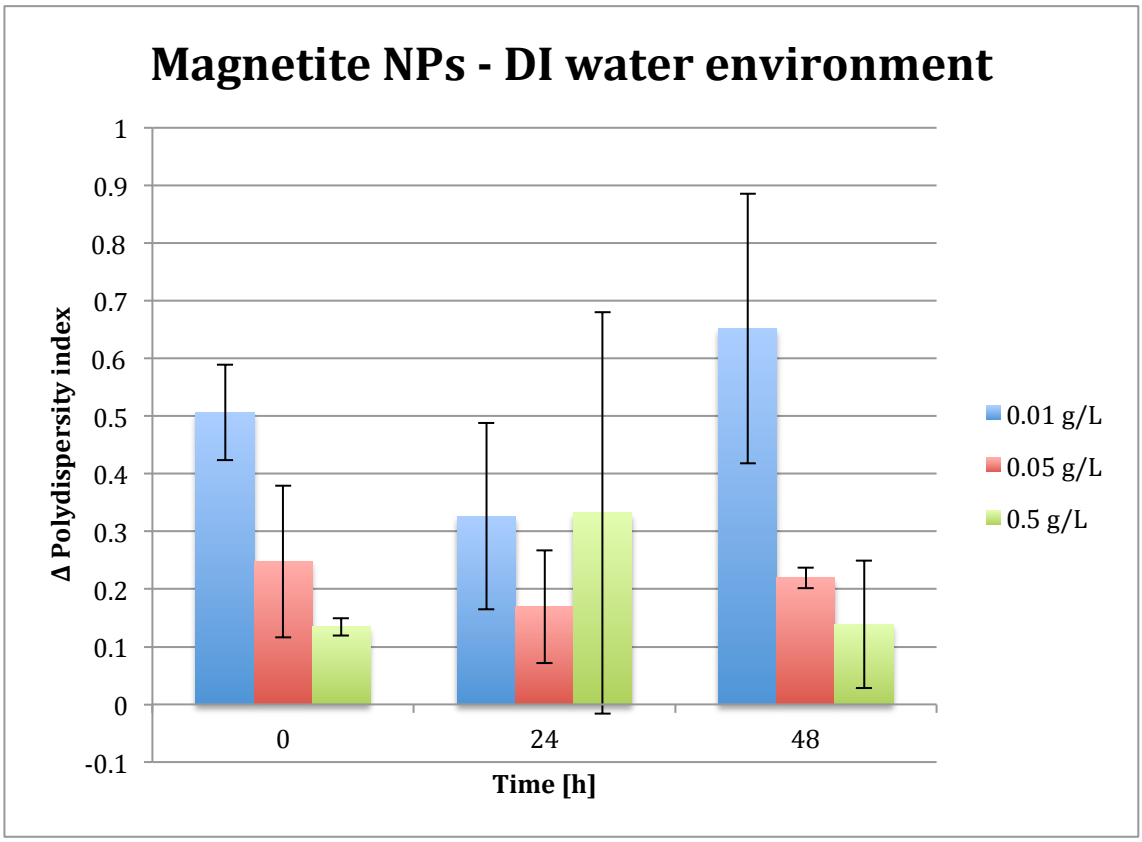

Nanodiamond - DI water environment
Δ Z-average [nm]
700
600
500
400
300
200
100
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

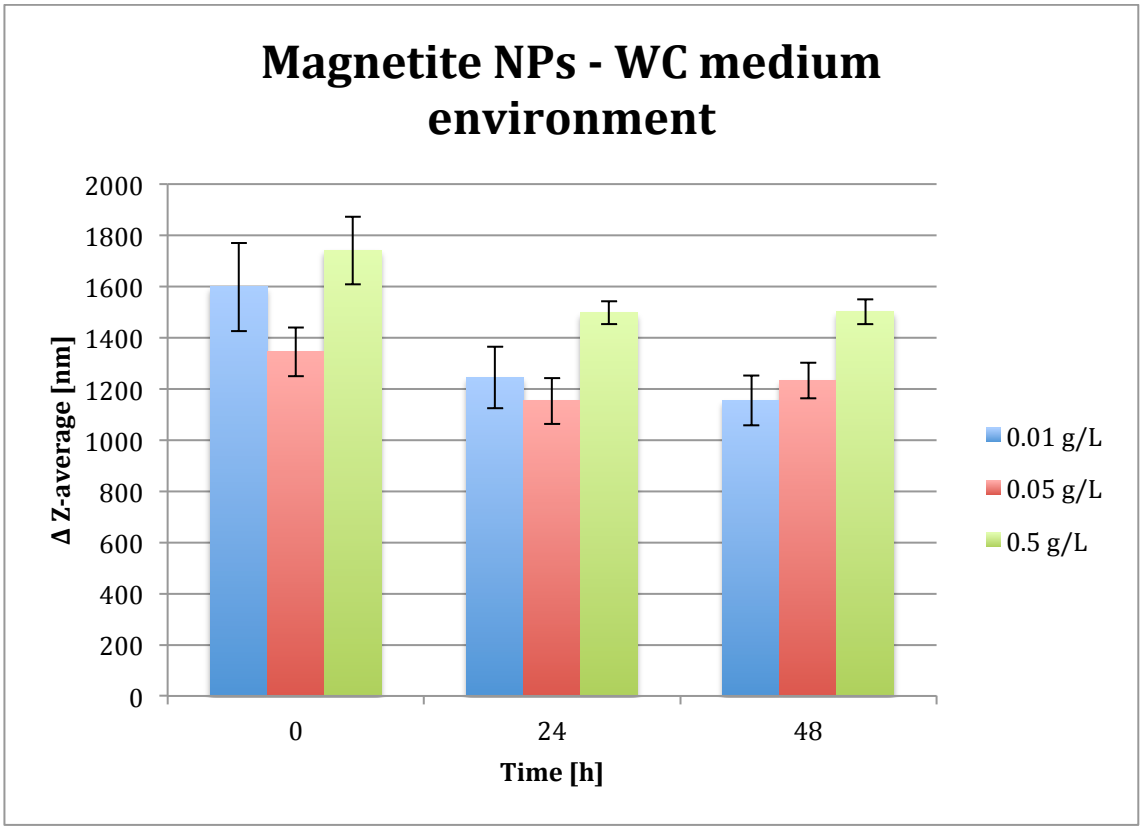

Nanodiamond - DI water environment
Δ Polydispersity index
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

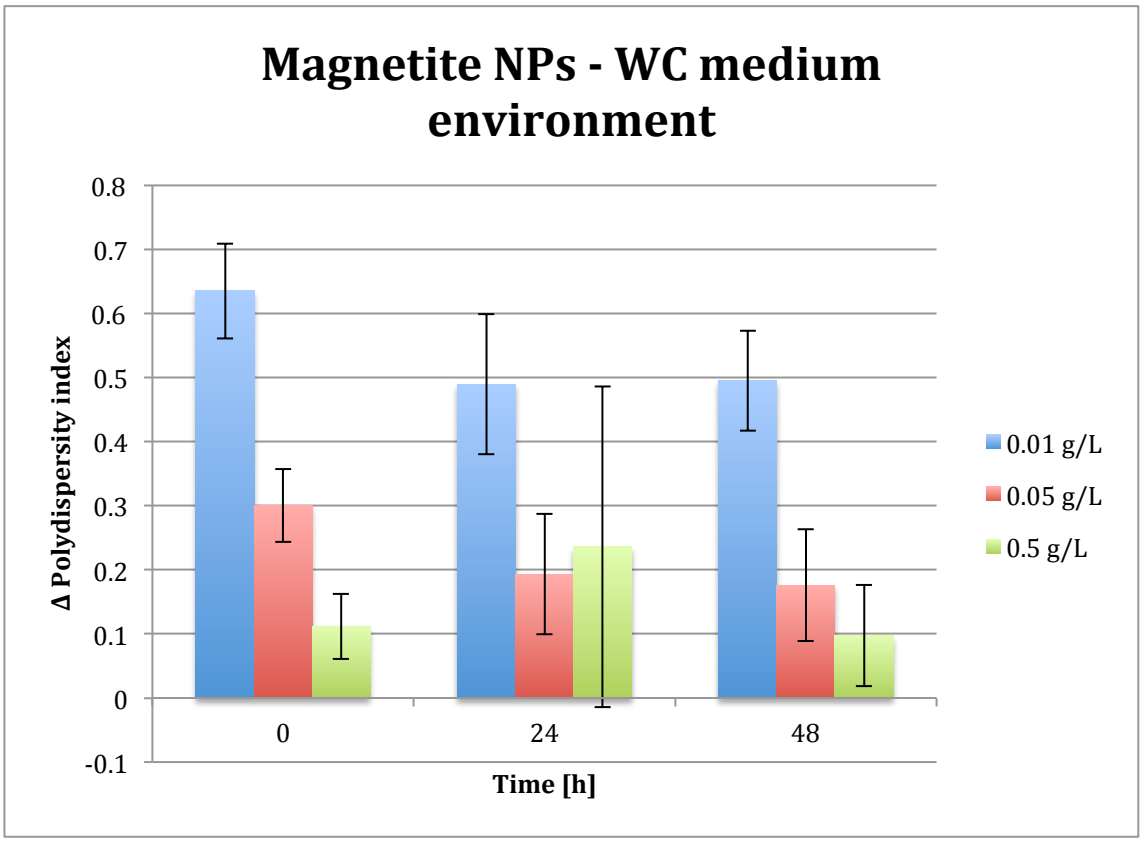

Nanodiamond - WC medium environment
Δ Z-average [nm]
3500
3000
2500
2000
1500
1000
500
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

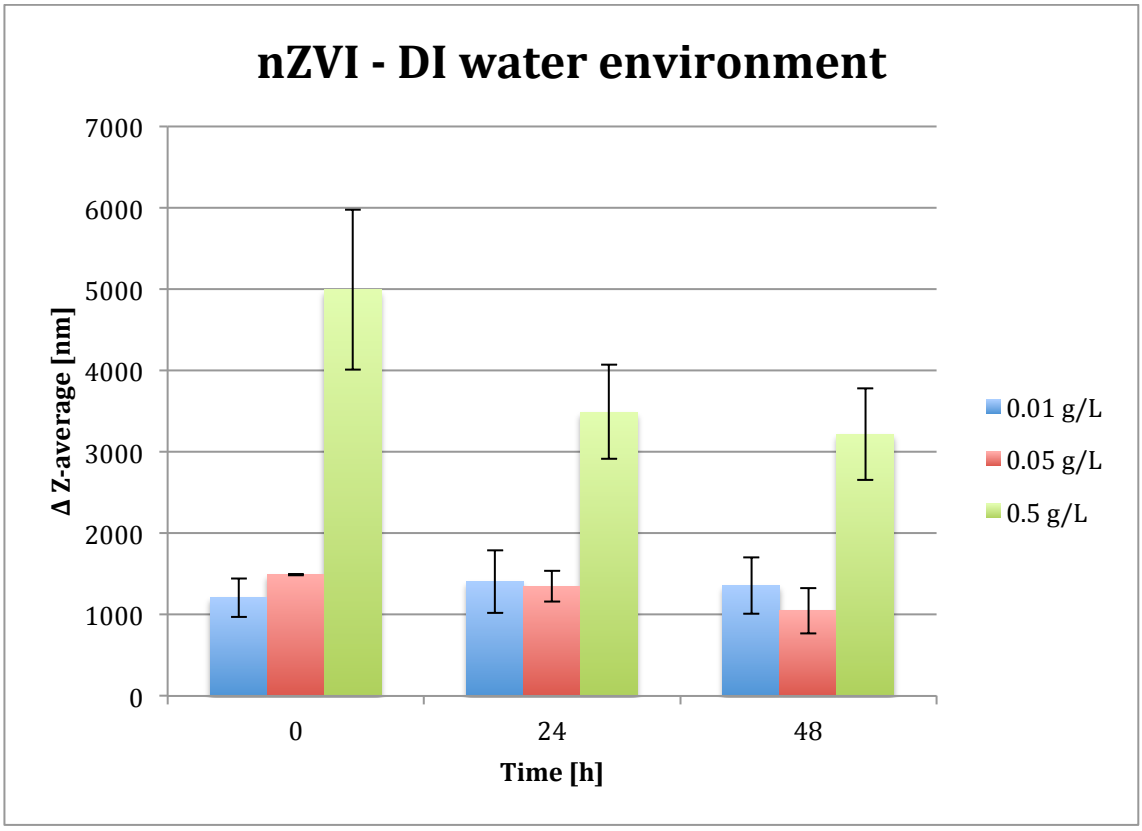

Nanodiamond - WC medium environment
Δ Polydispersity index
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

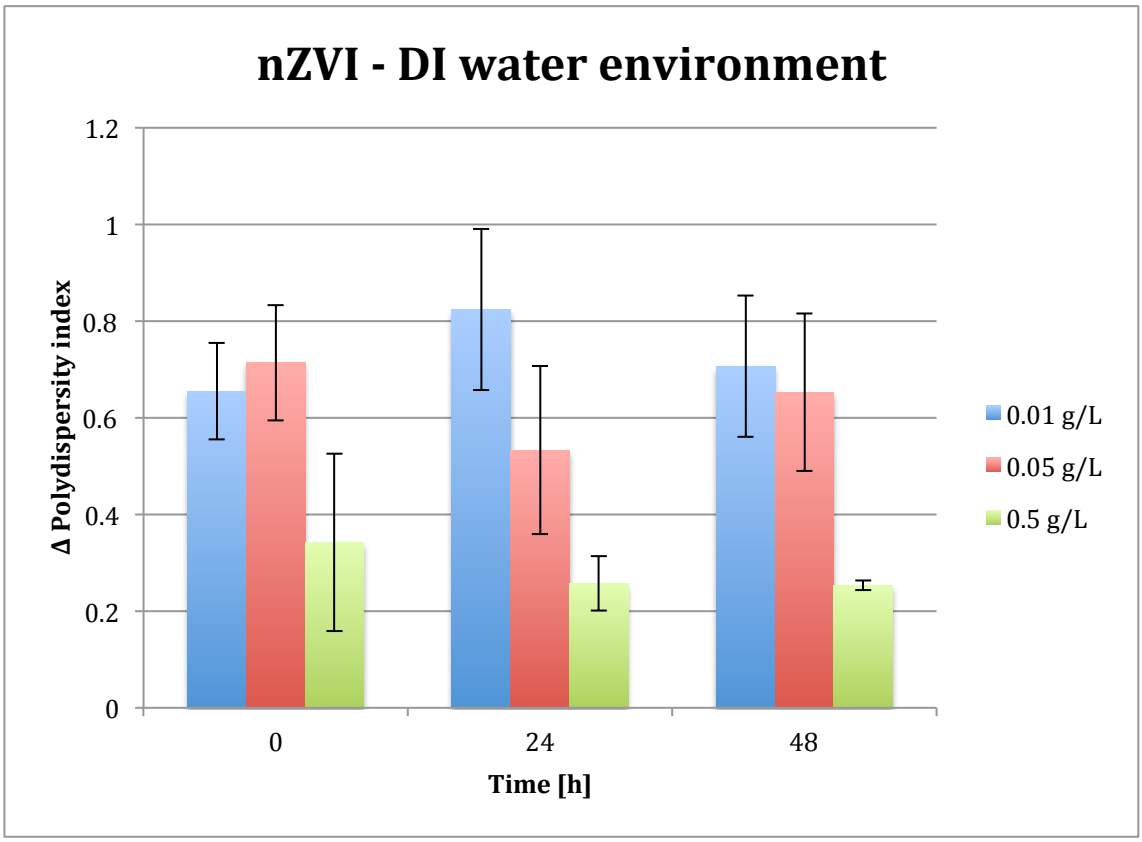

Magnetite NPs - DI water environment
3000
2500
2000
1500
1000
500
0
Δ Z-average [nm]
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

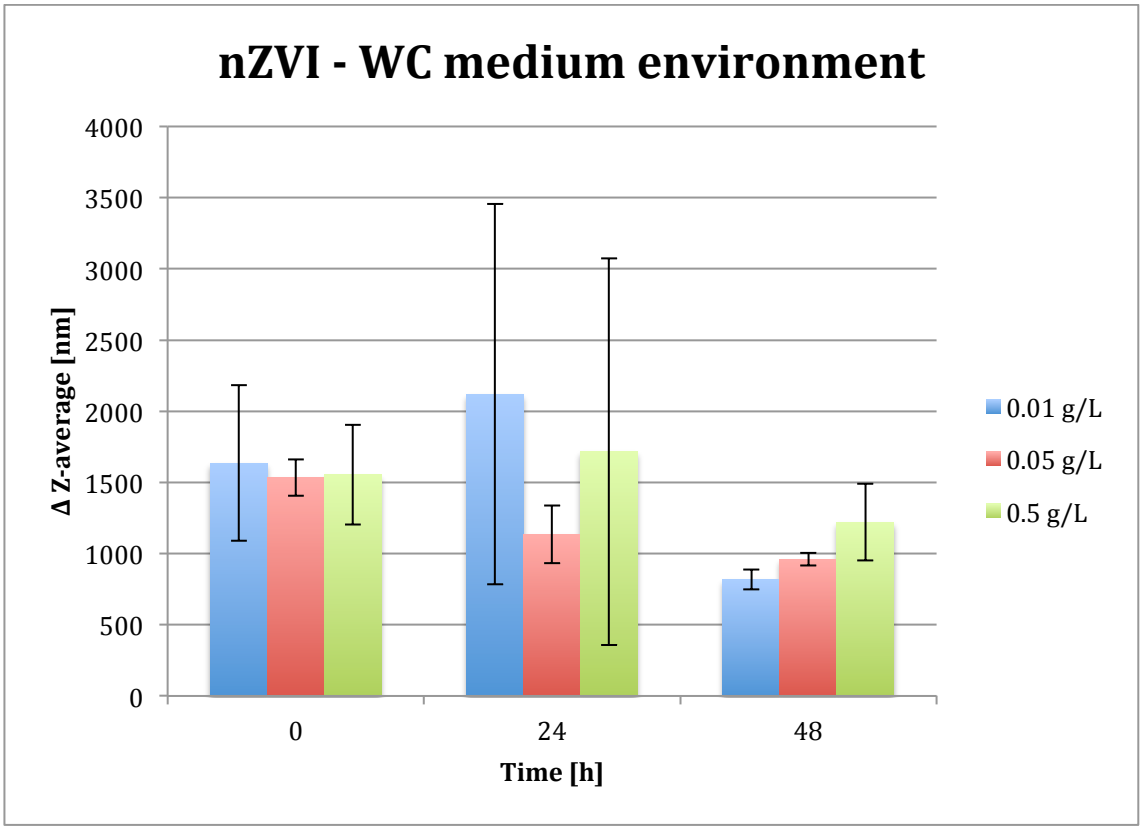

Magnetite NPs - DI water environment
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Δ Polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

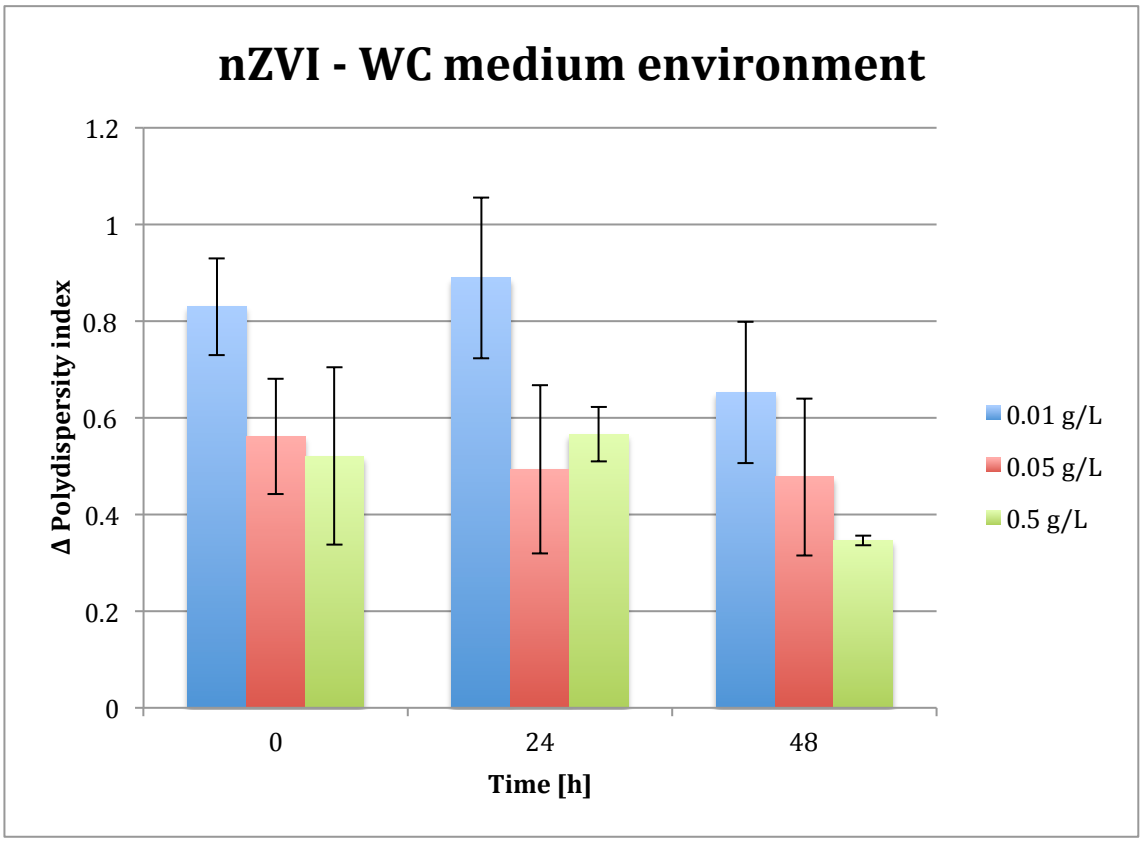

Magnetite NPs - WC medium environment
Δ Z-average [nm]
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


Magnetite NPs - WC medium environment
Δ Polydispersity index
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


nZVI - DI water environment
Δ Z-average [nm]
7000
6000
5000
4000
3000
2000
1000
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


nZVI - DI water environment
Δ Polydispersity index
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


nZVI - WC medium environment
4000
3500
3000
2500
2000
1500
1000
500
0
Δ Z-average [nm]
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L


nZVI - WC medium environment
1.2
1
0.8
0.6
0.4
0.2
0
Δ Polydispersity index
0
24
48
Time [h]
0.01 g/L
0.05 g/L
0.5 g/L

# Appendix C

Attached CD contents:

- Bachelor thesis text
  - bachelor_thesis_2015_Filip_Hrncirik.pdf
- Photographs of measuring equipment
  - Aquapen_C_AP_C_100_PSI.jpg
  - Axioimager_fluorescence_microscope_ZEISS.jpg
  - disc_centrifuge_CPS_instruments.jpg
  - Synergy_HTX_Multi_mode_reader_Biotek.jpg
  - Zetasizer_Malvern_Instruments.jpg
- Photographs of cells
  - demonstration_of_SYTOX_on_positive_control_sample.jpg
  - living_cells_without_distrupted_cell_membrane.jpg
  - malfunction_in_nanodiamond_negative_control_sample.jpg
- Data sheets
  - characterization_of_nanoparticles.xlsx
  - comparison_of_different_methods.pzfx
  - statistical_analysis_of_toxicity_of_nanoparticles.pzfx